Molecular analysis of DNA damage induced by a novel trinuclear platinum complex (BBR 3464)

Thesis

UNRESTRICTED

MOLECULAR ANALYSIS OF DNA DAMAGE INDUCED BY A NOVEL TRINUCLEAR PLATINUM COMPLEX (BBR 3464)

Gennaro Giovanni Domenico Colella, Degree in Biological Sciences

Thesis submitted to the Open University for the degree of Doctor of Philosophy

Discipline of Life Sciences

March 2001

Istituto Nazionale per lo Studio e la Cura dei Tumori, Milan-Italy

DATE OF SUBMISSION : 3 APRIL 2001
DATE OF AWARD : 16 JULY 2001

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ABSTRACT

We evaluated the activity of the trinuclear platinum complex BBR 3464 in two human ovarian carcinoma cell lines (OAW42, A2780) and in their cisplatin-resistant counterparts (OAW42Mer, A2780cp8). An increased cytotoxic potency of BBR 3464 compared to cisplatin was generally observed, an a collateral sensitivity or a very modest cross-resistance to BBR 3464 was found in OAW42Mer and A2780cp8 cell lines, respectively. Loss of mismatch repair proteins (hMLH1, hPMS2) or overexpression of nucleotide excision repair proteins (ERCC1) was not detrimental for the cellular sensitivity to the trinuclear platinum complex. BBR 3464 intracellular accumulation and DNA-bound platinum were consistently higher than those observed with cisplatin. After exposure to BBR 3464 and cisplatin of purified DNA or intact cells, a similar sequence preference of DNA damage was observed. Conversely, interesting differences in the kinetics of formation and removal of DNA lesions at the single-gene *(N-ras)* level were observed between the two drugs.

The interference exerted by BBR 3464 with cell cycle progression and its ability to induce apoptosis were evaluated in OAW42 and OAW42Mer cell lines. Flow cytometric experiments indicated that in the two cell lines BBR 3464 was able to induce a persistent G_2M block whereas cisplatin caused an initial accumulation of cells in the S phase followed by an increase in the G_2M cell fraction. Exposure to IC_{50} drug concentrations induced apoptosis in both cell lines. However, the percentage of cells with an apoptotic nuclear morphology was slightly higher after cisplatin than BBR 3464 treatment in OAW42 cells, whereas the opposite pattern was observed in OAW42Mer cells.

Degradation of the nuclear lamin B was detected in OAW42 cells after exposure to each drug whereas in OAW42Mer cells the cleavage was only appreciable after BBR 3464 exposure. In OAW42 cells the mitochondrial membrane potential ($\Delta\psi_{mt}$) was not affected by the two drugs, whereas in the OAW42Mer cell line a marked $\Delta\psi_{mt}$ reduction was observed only after exposure to BBR 3464.

Overall, the results would suggest that the collateral sensitivity to BBR 3464 observed in the OAW42Mer cell line might be attributable to the ability of this drug to modify DNA differently from that of cisplatin and, as a consequence, to induce different cellular responses to DNA damage such as the triggering of specific apoptotic pathways.

ACKNOWLEDGEMENTS

The work in this thesis was performed under the supervision of Dr Nadia Zaffaroni at the Unità Operativa n. 10 "Determinazioni Bio-Molecolari nella Prognosi e Terapia dei Tumori"-Dipartimento di Oncologia Sperimentale of the Istituto Nazionale per lo Studio e la Cura dei Tumori of Milan.

I am extremely grateful to the Dr Nadia Zaffaroni, who has been a constant source of guidance and support and who gave much of his time and interest to this project. I would like to thank also the Director of the Unità Operativa n. 10, Dr Maria Grazia Daidone, who gave me the possibility to frequent the laboratories to develop this project.

I am grateful to many colleagues at Unità Operativa n. 10. In particular, I would like to thank:

Dr Marzia Pennati and Dr Linda Orlandi for their advice and supports in performing cellular and molecular biology experiments;

Dr Alessandra Bearzatto for her advice and support in performing molecular biology experiments and for the revision of the manuscript for the English language;

Miss Gabriella Abolafio for her technical assistance in flow cytometry analyses.

In addition, I would like to thank:

Dr Carla Manzotti (Novuspharma), who kindly supplied the BBR 3464 compound;

Dr Roberto Leone (Università degli Studi of Verona) for the coupled plasma mass spectroscopy determinations of platinum bound to genomic DNA;

Dr Donato Colangelo (Università degli Studi of Torino) for the atomic absorption spectrometry determinations of intracellular drug accumulation;

Dr Massimo Broggini (Mario Negri Institute of Milan) who kindly supplied the HCT-116 cell line;

Dr R.A. Britten (Department of Radiation Oncology, University of Liverpool) who kindly supplied the OAW42 and OAW42Mer cell lines.

Finally, I would like to thank Professor Ian Hart, who acted as my English supervisor, for his advice, support and kindness, and his secretary, Mrs Fiona Parkinson, for the revisions of the thesis proofs.

This thesis is dedicated to my wife Gabriella.

LIST OF FIGURES AND TABLES

INTRODUCTION

(1)

1.1 Anticancer Drugs

Several therapeutic possibilities are available for the treatment of cancer: surgery, radiotherapy and chemotherapy. As regards pharmacological approaches in cancer therapy, increased knowledge of tumor biology obtained in the last 15 years with the identification of oncogenes, tumor suppressor genes, cell cycle, cell death and DNA repair genes, has opened important targets for the discovery/design and synthesis of novel anticancer drugs with improved antitumor activity.

At present, about 30 drugs are used in chemotherapy. These compounds are classified into different groups on the basis of their mechanism of action.

Antimetabolites, which are structural analogues of enzymatic cofactors or nucleosides, include inhibitors of folate synthesis, uracil analogues, cytidine analogues and purine analogues. These molecules are able to inhibit the synthesis of DNA precursors and/or can be themselves incorporated into nucleic acids, thus altering DNA replication and repair processes.

Another important class of anticancer drugs is represented by the poisons of the DNA topoisomerase I and II enzymes. By covalently binding the binary complex DNA-topoisomerase I, camptothecins are able to produce a 'cleavable' ternary complex of DNA-topoisomerase I-drug in which topoisomerase I activity is blocked. The presence of such cleavable complexes on genomic DNA seems to interfere with the DNA replication machinery thus altering normal DNA synthesis. Other drugs, such as DNA-topoisomerase II poisons

doxorubicin, daunorubicin, mitoxantrone and etoposide are able to stabilise the binary complex DNA-topoisomerase II forming a ternary drug-DNA topoisomerase II complex, thus leaving on the cellular DNA highly toxic double-strand breaks.

Molecules which exert their cytotoxic activity acting on the mitotic spindle assembly or disassembly are classified as antimitotic drugs. Compounds such as vinblastine, vincristine and vinorelbine are able to prevent mitotic spindle formation by inhibiting microtubule polymerisation, whereas taxanes act by inhibiting microtubule depolymerisation thus stabilising the mitotic spindle.

Alkylating agents represent a class of anticancer drugs actively used in the clinical approach to cancer therapy. These compounds are known for their ability to become strong electrophilic intermediates able to covalently interact with nucleophilic centres (phosphate, aminic, sulphydrilic, hydroxylic, carboxylic and hymidazolic groups) located on proteins and nucleic acids. It has been demonstrated that the most important target of such drugs is the genomic DNA. Within it, the most frequently alkylated sites are N^7 and O^6 of guanine, N^7 and N^3 of adenine and N^3 of cytosine. Several studies performed in the last few years, have provided evidence that these drugs exert their cytotoxic activity by altering the physiological DNA metabolism, in terms of replication, transcription and repair processes, thus producing molecular signals that can trigger apoptosis.

For many years it has been thought that alkylating agents targeted DNA in a random fashion whereas recent investigations have shown that the DNA-

alkylation produced by these compounds is made with a certain degree of sequence specificity. Major groove alkylators, such as nitrogen mustards (for example, melphalan) and alkylsulfonates (for example, busulfan), covalently bind N^7 only of guanines located in a run of guanines (5'-GGG-3') thus producing interstrand cross-links. Minor groove alkylating agents, such as benzoyl mustard tallimustine and CC-1065, covalently bind to N^3 of adenines with the highest sequence specificity ever seen for alkylators: in fact, these agents are able to alkylate only the N^3 of adenines located in the 5'-TTTTGA-3' and 5'-PuNTTA-3' or 5'-AAAA-3' respectively (Hurley et al., 1984; Broggini et al., 1991, 1995; Colella et al., 1996).

Cisplatin and its derivatives could be, to a certain degree, also considered as major groove alkylators, although their molecular structures are different from those of classical alkylating agents described above. Platinum-based anticancer agents are able to covalently bind N^7 of guanine with a certain degree of sequence selectivity thus producing various types of DNA adducts.

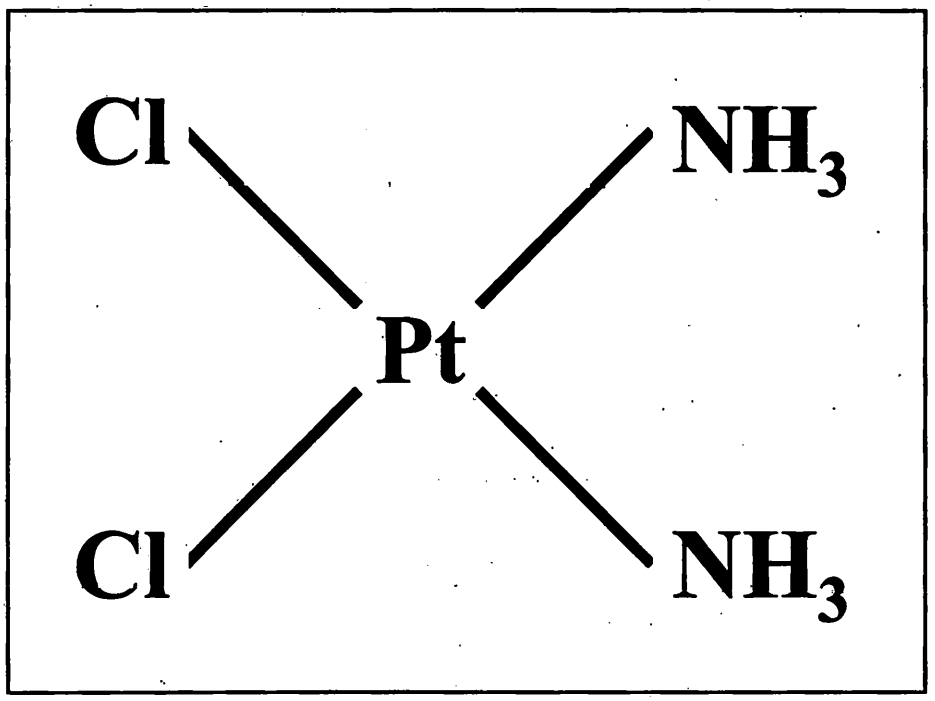
1.2 Cisplatin: properties and mechanism of action

Platinum coordination complexes have been known for many years. Cisplatin [cis-diammne-dichloroplatinum (II)], a bifunctional DNA damaging agent commonly used in cancer chemotherapy alone or in combination with other anticancer agent, was synthesised first in 1845 as Peyrone's chloride and

its planar structure was deduced in 1893. Interest in the biological effects of cisplatin was stimulated in 1965 during studies on the effect of the electric current on E. coli. In these experiments cell division was inhibited not by electric current but also by the production of cis-diamminodichloroplatinum (II) from the platinum electrodes. Cisplatin was shown subsequently to have activity against a variety of experimental tumors, and today it is firmly established as a highly potent anticancer agents for the treatment of many malignancies, including testicular, ovarian, head and neck, bladder, germ cell, esophageal, and small cell lung cancers (Ozols and Young, 1991; Dancey and Le Chevalier, 1997; Loehrer. and Einhorn, 1984).

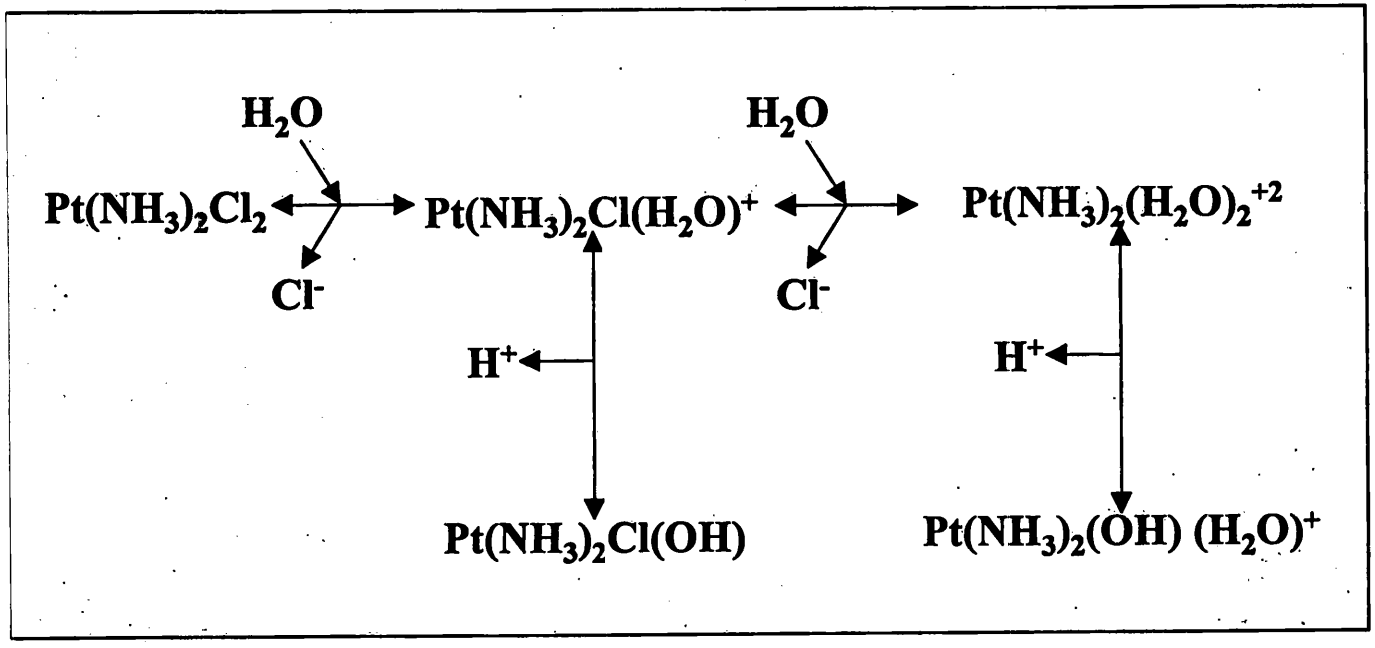
Cisplatin is a neutral square-planar coordination compound (Fig. 1). The two chloride ligands are stable at extracellular chloride concentration, but after diffusion into a cell, the lower chloride concentration therein facilitates exchange of the chloride ligands for water (Fig. 2). In fact, after intravenous administration cisplatin is relatively less reactive in the extracellular space, where the chloride concentration is about 100 mM, but, upon crossing the cellular membrane, it is activated in the intracellular space where the chloride concentration drops to about 3 mM. This exchange produces a potent bifunctional, positively-charged electrophilic aquated species, which represent the reactive form of the compound. The reactive product is able to react with any negatively charged-nucleophilic sites in the cell, including sulphydrilic, hydroxylic, carboxylic, hymidazolic, amminic and phosphate groups located on proteins and nucleic acids (Chu, 1994).

Figure 1. Chemical structure of cisplatin.



-Figure 1-

Figure 2. Hydration reaction of cisplatin. When cisplatin is dissolved in aqueous solution, chloride ions are displaced to allow the formation of aquated species, which are the reactive forms of the drug.


H2O
H2O
Pt(NH3)2Cl2
Pt(NH3)2Cl(H2O)+
Pt(NH3)2(H2O)2+2
Cl-
Cl-
H+
H+
Pt(NH3)2Cl(OH)
Pt(NH3)2(OH) (H2O)+

-Figure 2-

The cytotoxic activity of cisplatin has been explained by a number of properties, including penetration of cellular membranes and accumulation in the cell and nucleus. Although this compound can interact with many structures in the cell, such as membranes, proteins, RNA and small thiol compounds (glutathione, cysteine, methionine), it has been extensively demonstrated that genomic DNA is the critical target for cisplatin cytotoxic activity. In this regard, it was observed that about 1% of the total amount of the drug which penetrates the cell combines with cellular DNA in the nucleus thus producing very cytotoxic platinum-DNA coordination complexes (Eastman, 1990; Chu, 1994). Supporting this hypothesis there are some observations which indicate that cell lines unable to inactivate cisplatin by intracellular detoxification systems and/or to remove cisplatin-induced DNA damage are markedly more sensitive to this drug (Fram et al., 1990; Mistry et al., 1991; Kasahara et al., 1991; Kelland et al., 1992c; Johnson et al., 1997)

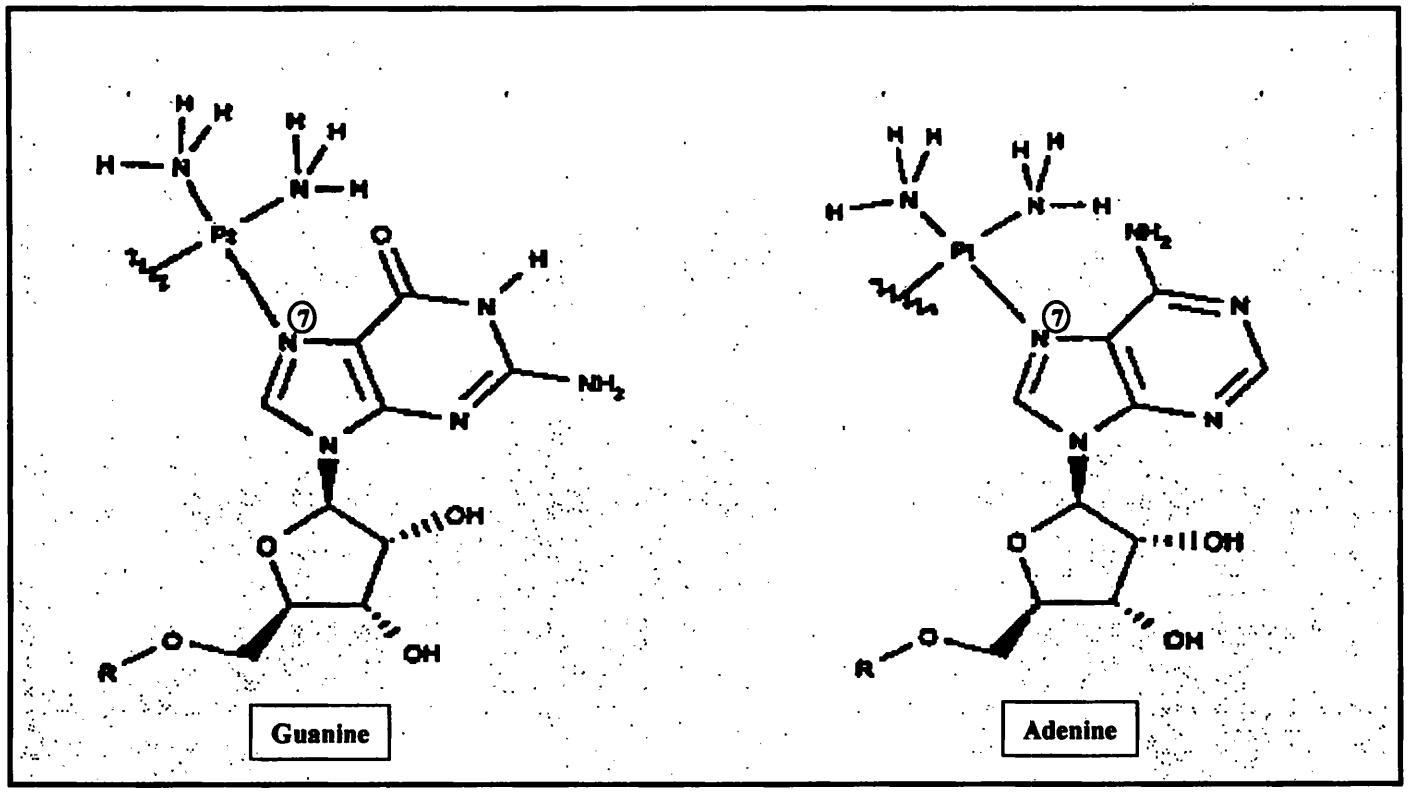
As mentioned above, the cytotoxic activity of cisplatin is believed to be due to its ability to covalently bind nucleophilic reactive centres located on DNA and proteins. By a mechanism somewhat as demonstrated for alkylating agents, the formation of platinum-DNA complexes is achieved by a reaction formed by both an activation and a nucleophilic attack phase. By an SN1 kinetic reaction, the DNA-binding drug is activated by cellular metabolism processes in a reactive form which is able to attack nucleophilic groups via SN2 kinetics reaction. By means of several biochemical, biophysical and molecular biology approaches, it has been demonstrated that nucleophilic centres covalently

bound by cisplatin are nitrogen atoms on position 7 of purinic bases (N^7-Guanine and N^7-Adenine) (Shin-Ichi Akiyama et al., 1999) (Fig. 3).

The lesions produced by cisplatin reacting with DNA are DNA-protein cross-links, interstrand DNA cross-links and intrastrand DNA cross-links, and all of these occur in the major groove of DNA. Quantitative studies show that DNA-protein cross-links account only for 1% of the cisplatin adducts formed *in vitro* whereas cross-links involving two nucleotides located on the same strand (intrastrand cross-links) or on the two opposite strands (interstrand cross-links) represent the most frequent cisplatin lesions (85-95%). Among these, the major lesion is a cross-link between two neighbouring guanines (1,2-intrastrand d(GpG)) which represents about 65% of the lesions both in experiments with purified DNA and in cells incubated with cisplatin. A further 25% of the lesions are cross-links between a neighbouring adenine and a guanine [1,2-intrastrand d(ApG)] and a neighbouring guanine and an adenine [1,2-intrastrand d(GpA)]. The remaining intrastrand cross-links are between two guanines separated by one or more bases [1,3-intrastrand g(GpNpG)]. Cisplatin also produces monofunctional lesions although some may occur as transient intermediates: in fact, cisplatin firstly forms monofunctional adducts with DNA, most of which are then converted into bifunctional adducts such as DNA-protein, DNA intrastrand and DNA interstrand cross-links (Crul et al., 1997) (Fig. 4).

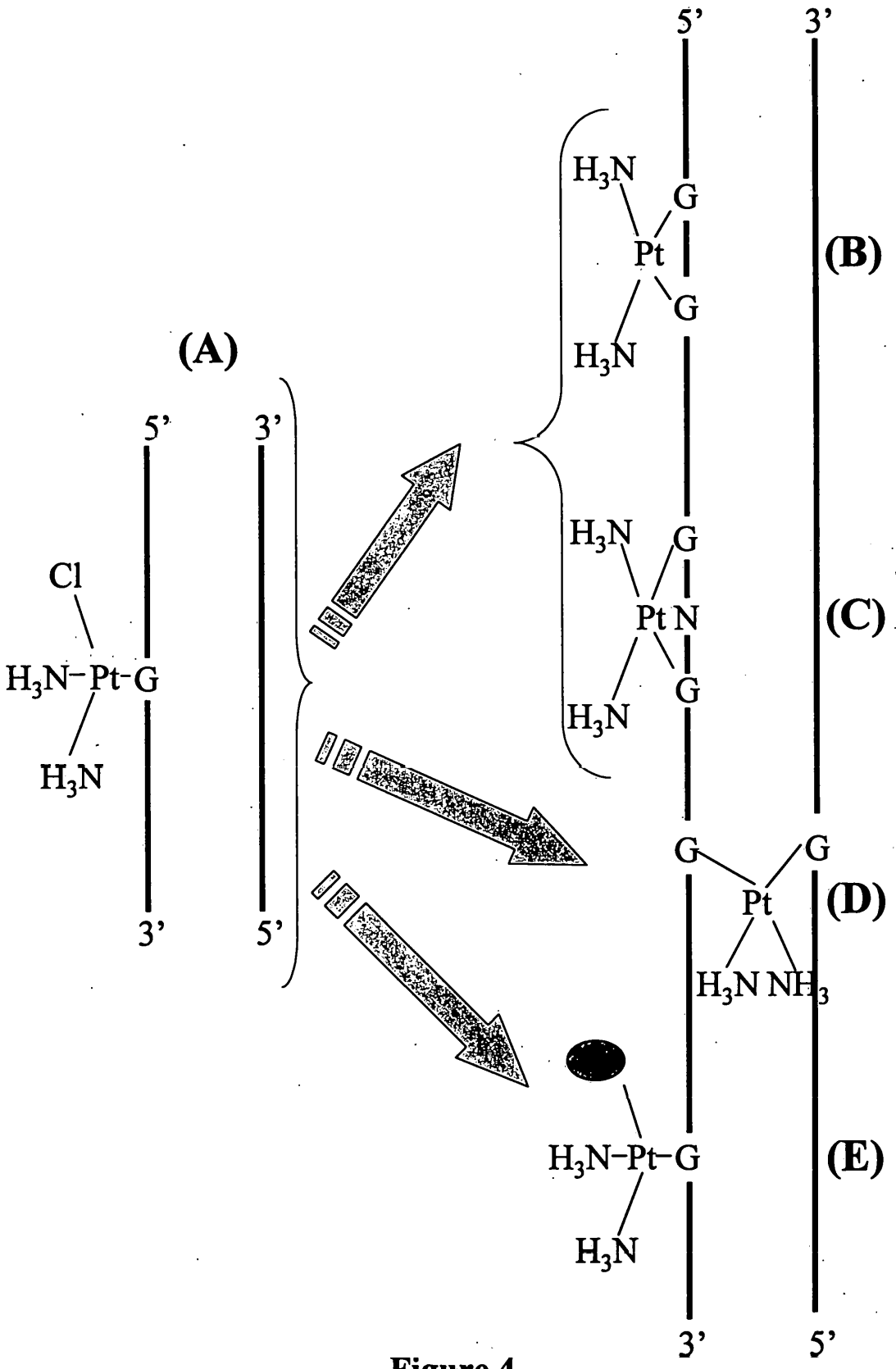
The therapeutic effects of cisplatin on human cancers may occur owing to several mechanisms. It has been demonstrated that by producing coordination

Figure 3. Nucleophilic sites located on DNA bases preferentially attached by cisplatin. The highly-reactive electrophilic intermediates derived from cisplatin activation are able to covalently bind nucleophilic groups such as the N^7 of guanines and adenines (DNA nucleotides).



-Figure 3-

Figure 4. Cisplatin DNA adducts. **(A)** Cisplatin bound monofunctionally to guanine; **(B)** 1,2-(GpG)-intrastrand cross-links; **(C)** 1,3-(GpG)-intrastrand cross-links (N represents a base); **(D)** interstrand cross-link; **(E)** cisplatin guanine-protein cross-link.


(A)
5'
3'
Cl
H3N–Pt–G
H3N
3'
5'
(B)
H3N
G
Pt
G
H3N
5'
3'
(C)
H3N
G
Pt
N
G
H3N
(D)
G
G
Pt
H3N NH3
(E)
H3N–Pt–G
H3N
3'
5'

-Figure 4-

complexes with DNA, cisplatin is able to inhibit DNA replication and chain elongation (Eastman, 1990). In fact, X-ray diffraction studies performed on a cross-linked dinucleotide [cis-$Pt(NH_3)_2$d(GpG)] indicated that the two guanines were completely destacked, and the deoxyribose sugar of the 5'-deoxyguanosine is in a C3'-endo pucker. Thus the intrastrand cross-link produces a severe local distortion in the DNA double helix, leading to unwinding and kinking (Bellon et al., 1991) which could be responsible for the alteration of the physiological metabolism processes of the DNA.

DNA damage caused by cisplatin seems to be the main cause of the antitumor activity of the drug, although the binding of cisplatin to DNA is not by itself sufficient to cause cell death. Cisplatin is much more toxic to dividing cells than resting cells, suggesting that some cell-cycle associated events, such as DNA replication and transcription, are required for its cytotoxic activity (Fraval and Roberts, 1979). Although its action is not dependent on a specific phase of the cell cycle, cisplatin can be up to 10 times more toxic to cells that are about to enter into S phase. Moreover, its toxic effect is a function of the amount of DNA damage remaining at the time cells enter S phase. These observations, together with the finding that DNA synthesis is inhibited in a variety of cells following platination, have led to speculation that inhibition of DNA replication may be the critical step in cisplatin toxicity, although some experimental results indicated that this hypothesis is not always true. Chinese hamster ovary cells (CHO), which are defective for a DNA repair pathway based on nucleotide excision repair (NER), and which are highly sensitive to

cisplatin, were found to progress through S phase at a normal rate following exposure to the drug; these cells then arrested in G_2 phase and subsequently died. DNA repair-competent cells could survive incubation with higher concentrations of cisplatin that inhibited DNA replication and slowed passage through the S phase. These cells also arrested in G_2 following exposure to the drug. The fate of these repair-competent cells was dependent on the concentrations of cisplatin. At low concentration of the drug, the G_2 arrest was transient and the cells eventually recovered, whereas at more toxic concentrations of the drug, the cells died. These data suggested that inhibition of DNA synthesis is not the critical step in cisplatin–induced cytotoxicity, and that essential events occur during G_2 phase which determine the fate of the cells.

It should be emphasised that although G_2 arrest appears to be a prerequisite for cell death (except at very high drug concentrations), not all such arrested cells died. At minimally toxic concentrations of cisplatin, cells may eventually bypass the block and return to normal cycling. Hence, there are two possible fates for a G_2-arrested cell: survival or death. It has been demonstrated that G_2 arrest in radiation-damaged yeast is controlled by a gene of the NER system called RAD 9 (Weinert and Hartwell, 1988). Cells deficient in the Rad 9 gene product fail to arrest in G_2 following DNA damage; instead they continue to cycle and then die (Weinert and Hartwell, 1988). An analogous system exists in mammalian cells, and this system may be relevant to the repair of DNA lesions induced by cytotoxic drugs such as cisplatin. A similar behaviour was observed

in cells lacking DNA mismatch repair (MMR) activity: in fact, conversely the situation observed in MMR-proficient cell lines, no G_2-arrest but just a G_1 delay was seen in MMR-deficient lines after exposure to alkylating agents and cisplatin (Hawn et al., 1995; Carethers et al., 1996).

Several investigators reasoned that the movement of the replication fork in cisplatin-treated cells would be inhibited selectively at sites of DNA damage, perhaps producing single strand gaps at the corresponding position in the daughter strand. It has been postulated that G_2 arrest could be a period during which such breaks would be repaired, a process often referred to as post-replicational repair (Eastman, 1990). Analysis of the DNA isolated from CHO cells after exposure to cisplatin indicated that it only contained double strand breaks (but not single strand breaks) and that these breaks occurred at a regular frequency within the chromatin (Sorenson, 1990), showing a typical pattern of the endonucleolytic digestion of chromatin DNA in the internucleosome spacer regions. These breaks became evident after the cells had progressed to, and arrested in, G_2 and their appearance correlated with toxicity. The breaks were not seen to be a consequence of cell death, since they occurred one day before NAD and ATP pools were depleted and two days before loss of membrane integrity. The overall levels of total RNA, mRNA, and protein synthesis did not decrease before the DNA breaks appeared, indicating that lethally injured cells continue to undergo apparently normal metabolic activity for several days. Both DNA digestion and cytotoxicity could be prevented by incubation of the cells with the protein synthesis inhibitor cycloheximide. These features are

characteristic of programmed cell death (also called apoptosis) and the data altogether indicated that DNA lesions caused by cisplatin can trigger apoptosis via G_2 arrest of the cell cycle progression (Anthoney et al., 1996; Sorenson and Eastman, 1988; Sorenson, 1990).

It has been postulated that DNA damage-dependent apoptosis is not the only way by which cisplatin can cause its antitumoral activity. As mentioned above, after exposure to this drug G_2-arrested cells can die by apoptosis or survive by removing platinum-induced lesions present on DNA. Cisplatin-DNA adducts may be qualitatively and quantitatively similar in some cell lines but the ability of the cell to repair this damage appears dependent on the amount of repair enzymes as well as the availability of deoxynucleotides such as thymidine which can be supplied by folate metabolism and the dTMP synthase cycle. It has been demonstrated that cisplatin can influence both methionine and folate metabolism (Scanlon et al., 1983; Scanlon et al., 1986; Scanlon et al., 1988; Lu et al., 1988; Kashani-Sabet et al., 1988) and that there is a correlation between cisplatin's toxicity and exogenous folate requirements for optimal human cancer cell growth. In fact, when higher cisplatin concentrations are necessary for cytotoxicity, lower exogenous concentrations of folinic acid are required for optimal cell growth. Thus, a cell line relying more on its endogenous folate metabolism rather than the exogenous component is less sensitive to the cytotoxic effect of cisplatin.

1.3 Tumor cell resistance to cisplatin

The therapeutic efficacy of cisplatin is limited due to important side effects such as nephrotoxicity, nausea, vomiting, myelosuppression and ototoxicity (Prestayco et al., 1979). However the main clinical obstacle to the success of clinical treatment with cisplatin is intrinsic or acquired cellular resistance to this compound. In fact, some common tumors are intrinsically resistant to platinum and the frequent development of resistance in responsive tumors can be responsible for the failure of the cisplatin curative therapy. Relatively little is known about intrinsic resistance, but mechanisms of acquired resistance have been studied extensively (Chu, 1994; Andrews and Howell, 1990, Timmer-Bosscha et al., 1992; Zamble and Lippard, 1995) particularly in human tumour cells lines which had developed *in vitro* cisplatin resistance. In most cases the levels of resistance are less than 50-fold although there are reports of up to 1000-fold resistance. Nevertheless, even a small increase in resistance of a tumour to cisplatin can be clinically important, since a large dose escalation leads to severe general toxicity.

The potential mechanisms responsible for this acquired cisplatin resistance suggested so far can be classified into two categories: those that lead to a reduction in the formation of cytotoxic DNA lesions and those that minimise their impact (Johnson et al., 1994). The first category includes decreased drug accumulation, increased drug efflux and enhanced intracellular drug inactivation by thiol-containing molecules such as metallothionein proteins and

non-protein glutathione (GSH) (Andrews and Howell, 1990; Timmer-Bosscha et al., 1992; Zamble and Lippard, 1995; Johnson et al., 1994). The second category includes enhanced DNA repair and/or increased tolerance to DNA damage (Andrews and Howell, 1990; Timmer-Bosscha et al., 1992; Johnson et al., 1994) and alterations of genes involved in the cell cycle control such as p53 (Zunino et al., 1997).

1.3.1 Intracellular drug accumulation

Reduced drug accumulation was observed frequently in cisplatin resistant cell lines obtained from tumours of different origin (human squamous carcinoma, human teratoma, murine leukemia and chinese hamster ovarian carcinoma) (Hamilton et al., 1989, Andrews, 1994; Teicher et al., 1987; Hill et al., 1990). Conversely, investigations performed on other cell systems (human lung and rat ovarian carcinoma cells) provided evidence that differences in drug uptake between resistant and sensitive cells were not present (Meijer et al., 1990a; Sekiya et al., 1989). A reduced intracellular accumulation could result either from reduced uptake or active efflux, or both. Decreased cisplatin accumulation was due in part to defective influx of the drug in 2008 human ovarian carcinoma cells (Mann et al., 1990), but the real mechanisms of cisplatin transport into cells are not yet understood. Cisplatin generally is believed to enter the cells through passive diffusion, since the rate limiting factor for platinum uptake is the concentration of the platinum compound and

the uptake of cisplatin is not saturatable (Hromas, 1987b). Moreover, the accumulation of cisplatin is not significantly inhibited by excess amounts of analogues of cisplatin, such as transplatin and carboplatin (Gately and Howell, 1993).

There is some evidence suggesting that cisplatin uptake is mediated by membrane proteins. It has been shown that benzaldehyde compounds inhibit the uptake of cisplatin, presumably by forming Schiff bases with membrane proteins (Dornish et al., 1989). Moreover, the NA-K ATPase inhibitor ouabain also inhibits cisplatin uptake by 50% (Andrews et al., 1991). However, it has been demonstrated that this pump itself does not directly transport cisplatin and that cisplatin accumulation is K-dependent (Andrews et al., 1991). These findings suggest that cisplatin accumulation is dependent on cell membrane potential. A model of cisplatin accumulation that accommodates most of the existing observations was formulated (Gately and Howell, 1993). In this model, about 50% of the initial rate of uptake is due to passive diffusion and the remaining 50% is due to facilitated diffusion through an, as yet, unidentified gated channel.

Cisplatin resistant cell lines are also frequently resistant to methotrexate and several metal salts, such as sodium arsenite, sodium arsenate, antimony potassium tartarate and cadmium chloride. It has been observed that cross-resistance to these agents by cisplatin resistant cells derived from human hepatoma and cervical adenocarcinoma resulted from multiple changes in the

plasma membrane proteins that bind them (Shen et al., 1998). There may be a high-affinity uptake system for arsenite, arsenate and cisplatin.

Enhanced active efflux of cisplatin was observed in the cisplatin resistant human epidermoid carcinoma KCP-4 and in some other cisplatin resistant cells (Mann. et al., 1990; Fujii. et al., 1994a; Fujii. et al., 1994b). Experiments performed using somatic cell hybrids to test whether cisplatin resistance is a dominant or a recessive trait indicated that the resistance phenotype was incompletely dominant in the intraspecific hybrid between cisplatin resistant KCP-4 cells and cisplatin sensitive HeLa D98OR-1 cells (HATs, ouAr). KCP-4 cells showed a 70% decrease in cisplatin accumulation compared with the parental, cisplatin sensitive KB-3-1 cells, and the somatic cell hybrid showed a slightly higher cisplatin accumulation compared with KCP-4 cells when corrected for cell volume. These data indicate that defective accumulation is a co-dominant trait, and the data are consistent with the hypothesis that an active efflux pump for cisplatin is expressed in KCP-4 cells (Fujii et al., 1994a).

Cisplatin is not a substrate for P-glycoprotein GP-170 that is frequently overexpressed in multidrug-resistant cells and functions as a drug efflux pump (Gottesmann and Pastan, 1988).

The existence of an ATP-dependent glutathione S-conjugate export pump, named the GS-X pump, was hypothesised. This pump might efflux cisplatin conjugated with glutathione (GS-platinum complex) from L1210 murine leukemia cells (Ishikawa, 1992; Ishikawa et al., 1993) and as a consequence, plays a protective role by decreasing the intracellular concentration of the

complex. The presence of an active efflux system for cisplatin was detected in KCP-4 cells (Fujii et al., 1994a). These cells have an ATP-dependent transporter for leukotriene C4 (LTC4), a substrate for the GS-X pump (Fujii et al., 1994b). The LTC4 transporter was inhibited by the GS-platinum complex. Significant ATP-dependent uptake of GS-platinum complex was observed in membrane vesicles from KCP-4, but not in those from parental KB-3-1 cells or multidrug resistant C-A500 cells, which overexpress multidrug resistance protein (MRP) (Ueda et al., 1999). These findings suggest that the GS-X pump is involved in reducing the accumulation of cisplatin in KCP-4 cells (Chen et al., 1998).

It has been proposed that the overexpression of multidrug resistance-associated protein (MRP) and the increased level of GSH in cisplatin-resistant human leukemia HL-60 cells are responsible for cisplatin resistance (Ishikawa et al., 1996). However an MRP-transfected cell line that expressed high levels of MRP showed no cross-resistance to cisplatin (Cole et al., 1994). When the GSH levels in C-A120 cells that overexpressed MRP were increased to nearly the same levels as in KCP-4 cells by transfection with γ-glutamyl cysteine synthase (γ-GCS) cDNA, the resistance to, and the accumulation of, cisplatin in C-A120 cells were characteristics not significantly changed with respect to untransfected C-A120 cells (Chen. et al., 1998). These results indicate that MRP is not involved in cisplatin resistance.

Studies performed on cell lines resistant to both cisplatin and doxorubicin evidenced a positive association between canalicular multispecific organic

anion transporter cMOAT (also known as Multidrug Resistance-associated protein 2 MRP2) overexpression and cisplatin resistance.

Studies performed transfecting cMOAT antisense cDNA in human hepatic cancer cells HepG2 (that express cMOAT) (Koike et al., 1997) indicated that cMOAT is involved in the transport of cisplatin and vincristine and in resistance to both agents. However, neither MRP nor cMOAT were detected in KCP-4 cells (Chen, 1998) and the characteristics of the LTC4 transporter expressed in KCP-4 vesicles were similar but not identical to that of MRP (Chuman et al., 1996). These findings suggest that an efflux pump for cisplatin, different from MRP and cMOAT, is involved in cisplatin resistance in KCP-4 cells.

1.3.2 Cellular detoxification systems: glutathione and metallothioneins

It has been extensively demonstrated that detoxifying systems are important determinants of resistance to chemotherapeutic drugs based on their ability to neutralise cytotoxic activity of electrophilic agents such as cisplatin or quinone-containing agents like adriamycin.

Glutathione (GSH) is the most important non-protein thiol in living systems. It is known to function directly or indirectly in many important biological phenomena, including synthesis of proteins and DNA, transport (amino acids, ions and/or sugars), enzyme activity, metabolism and protection of cells (Arrick

and Nathan, 1984; Kosower. and Kosower, 1978; Meister and Anderson, 1983).

The biosynthesis of GSH proceeds via two successive ATP requiring steps, one catalysed by γ-glutamylcysteine synthase (γ-GCS) and the other catalysed by glutathione synthase (GS) respectively, thus producing a γ-glutamylcysteinylglycine thiol tripeptide. It is important to note that such a biosynthetic pathway is also regulated by GSH itself through feedback inhibition. Under normal physiological conditions, the majority of GSH in mammalian cells exists in the reduced form (0.5 to 10 mM). Oxidation of GSH yields glutathione disulfide (GSSG). NADPH-dependent reduction of GSSG by the enzyme glutathione reductase (GR), as well as efflux of GSSG, effectively maintains the cellular concentration of GSSG at very low levels (5 to 50 μM). Furthermore, cellular GSH also participates in mixed disulfides with both protein and non-protein sulphydryls and in thiol esters of GSH (Arrick and Nathan, 1984).

GSH plays a role in decreasing the toxicity of several drugs by facilitating their metabolism to less active compounds. It acts as a nucleophile towards a wide variety of electrophilic agents leading to so-called GSH conjugates. These conjugates are either excreted as such or are metabolised to mercapturic acids and excreted in either bile or urine. This irreversible loss of GSH requires synthesis of GSH to restore the cellular GSH level (Kosower and Kosower, 1978).

The interaction of electrophilic agents with GSH can proceed spontaneously or it can be catalysed by Glutathione-S-transferase (GST), a family of cellular isoenzymes. These isoenzymes have generally been identified by both characteristic isoelectric points and substrate specificities. So far, three cytosolic GST families have been identified on the basis of their isoelectric points in man. These have been termed α (basic), μ (neutral) and π (acid, anionic). As these isoenzymes are known to have markedly different substrate specificities, both the total GST activity and the isoenzyme composition may be important determinants of a tumour cells ability to detoxify specific antineoplastic compounds (Douglas, 1987; Kosower and Kosower, 1978; Mannervik and Danielson., 1988; Meister and Anderson, 1983). Thus, the effectiveness of this conjugation pathway depends upon the cellular GSH concentration, the presence of GST of appropriate specificity and/or the capacity of the cell to rapidly resynthesise GSH.

Along with direct interaction with drugs, xenobiotics and other chemical substances, GSH also plays a role in the detoxification of drug-induced free radical species. Free radicals may be formed physiologically, such as in metabolic processes in mitochondria, in microsomes and peroxisomes and could interfere with different cellular processes. Pathologically reactive oxygen intermediates may be generated by radiation, by elevated temperatures or by intracellular metabolism of chemical substances including antineoplastic agents like adriamycin, daunomycin, bleomycin, mitomycin C, which are activated to

semiquinone radicals by NADPH-dependent flavin reductase (Meijer et al., 1990a).

GSH is also essential for the synthesis of the DNA precursors deoxyribonucleoside triphophates (dNTPs). In fact, the synthesis of dNTPs from ribonucleotide diphophates is catalysed by ribonucleotide reductase through a system involving glutaredoxin, NADPH and GR. This may give GSH a function in the repair of DNA damage. The depletion of GSH may inhibit repair through the reduction of the dNTP pool size, although the requirement for repair synthesis would be small when compared to that needed for replicative DNA synthesis (Castellot et al., 1979; Holmgren, 1979; Luthman et al.,1979).

In addition to GSH, metallothioneins (MTs), may act as another nucleophile towards electrophilic agents. This family of 6-7 KDa proteins appears to serve a number of different biological purposes, such as zinc homeostasis and detoxification of heavy metals. In the case of exposure to electrophilic agents, the increased supply of MTs could provide the 'neutralising' nucleophilic equivalent since MTs, which contain 30% of cysteine residues (20 of 61 amino acids), are rich in nucleophilic thiol groups. Although the majority of these thiols are bound to zinc, it appears that they are still capable of reacting with cisplatin and thus may serve as intracellular "sinks" that inactivate incoming cisplatin. It is known that MT gene transcription is strongly induced by heavy metal ions, glucorticoids, interferon and stress, whereas the ability of cisplatin to induce MT expression is still unclear (Chu, 1994).

It has been demonstrated that cisplatin is able to bind MTs with a molar ratio of 10 platinum atoms for 1 MT molecule (much higher than that of GSH) and, when cisplatin binds to MT, it loses its amine ligands and displaces heavy metal ions as described by the following reaction:

$$(Zn^{2+})_7\text{-}MT + 10(NH_3)_2Pt_2 \rightarrow (Pt^{2+})_{10}\text{-}MT + 20(NH_3) + 7(Zn^{2+})$$

In the last few years it has been extensively demonstrated that cellular resistance to cisplatin is multifactorial and that one of the possible determinants of such a resistance can be an inactivation of the drug by cellular thiols such as GHS and MTs which are able to prevent the reaction between the cisplatin and cellular nucleic acids. Results from several studies performed have provided a correlation between cisplatin resistance and increased levels of GSH in several murine and human tumour cell lines (Hromas et al., 1987a; Richon et al., 1987; Mansouri et al., 1989; Beherens et al., 1987; Fram et al., 1990; Fujiwara et al., 1990; Hospers et al., 1988; Meijer et al., 1990b; Hamilton et al., 1985; Andrews et al., 1988; Andrews et al., 1989).

A direct correlation was observed between the degree of cisplatin resistance and GSH levels in a panel of human small cell lung carcinoma cell lines (hSCLC) (Hospers et al., 1990b). Moreover a close correlation between GSH and native cisplatin resistance has also been reported in human ovarian carcinoma cells (Mistry et al., 1990).

Cellular GSH can be depleted by administration of various compounds able to interact with GSH by forming conjugates and promoted by the action of glutathione S-transferases (GST). The most frequently used method to decrease GSH is via inhibition of glutathione synthesis by buthionine sulfoximine (BSO) (Griffith and Meister, 1979) which selectively interacts with γ-GCS, one of the two GSH synthesis enzymes. Long-term GSH depletion with BSO in murine mammary carcinoma P-66 cells, however, resulted also in an inhibition of protein and DNA synthesis and a reduction of cells in the S-phase (Dethlefsen et al., 1986). This cellular toxicity may well be cell-type dependent as in most studies dealing with GSH depletion by BSO no signs of toxicities are mentioned. Selectivity may be achieved in situations in which the tumour cells and the normal cells have different quantitative requirements for GSH.

The effect of BSO on the GSH content of a number of normal tissues and three experimental tumours was studied in female CH/HeJ mice (Lee et al., 1987). Considerable diversity in response to treatment with BSO was demonstrated. GSH depletion can be achieved in tumours with BSO doses lower than those required for normal tissues, with the exception of the lung. This supports the idea that the therapeutic index of some chemotherapeutic drugs can be improved by BSO.

It has been shown that when BSO is used to reduce GSH levels, the cytotoxic effect induced by cisplatin in tumour cell lines varies in a 22-fold cisplatin-resistant hSCLC cell line, with a 2,5-fold increase in GSH content compared to the parent cell line, exposure to BSO reduced cellular GSH content to a non-

detectable level and increased the cisplatin cytotoxic activity (Meijer et al., 1990b). Other studies, however, found no effect of BSO treatment on cisplatin-induced cytotoxicity (Richon et al., 1987; Teicher et al., 1987). A probable explanation for this is that a reduction of GSH content below a critical level, and perhaps more importantly, at certain subcellular compartments such as the nucleus, is required to obtain a marked sensitisation to this drug by BSO. Measuring the total GSH can, therefore, be misleading since the concentration of GSH in a particular compartment of the cell may be far more important than the average cellular concentration. Another reason for the observed discrepancies in the results may be the fact that the steady state of GSH is generally used to express the capacity of this defence system, without considering GSH biosynthetic capacity of individual cell lines.

The activity of the GSH-related enzyme GST may also be a factor in determining drug response. Overexpression of one or more GST isoenzymes has been found in a number of different tumour cell lines selected *in vitro* for resistance to alkylating agents (Bakka et al., 1981; Robson et al., 1987; Wang and Tew, 1985). GST was found to be elevated in a cisplatin resistant human squamous carcinoma cell line. The isoelectric point of the GST isoenzyme was 4.8 in both the resistant and the parental sensitive cell line, which suggests the induction in the resistant cells of the predominant isoenzyme present in the parental cell line (Teicher et al., 1987). In a cisplatin-resistant human melanoma cell line, a 5.4-fold increase in GST activity was associated with a 4.5-fold elevation of the GST-π isoenzyme protein consequent to a 15.2-fold

increase in mRNA levels (Wang et al., 1989). Also in a Chinese hamster ovary cell line, cisplatin resistance was associated with an increase in GST activity and GST-π mRNA. No apparent gene amplification of the GST-π gene was observed in resistant cells (Saburi et al., 1989). Also in this study, no increased GST activity was found in a cisplatin-resistant human prostate cancer cell line. Again, overexpression of GST-π in two GST-π-transfected cell lines did not influence cellular sensitivity to alkylators such as chlorambucil, melphalan and cisplatin (Nakagawa et al., 1990).

Inhibitors of GST activity, such as ethacrynic acid (EA), have successfully been used in a Walker-256 rat breast carcinoma cell line with acquired resistance to nitrogen mustards and in two human colon carcinoma cell lines, HT29 and BE (Tew et al., 1988). EA mediated inhibition of GST also increased melphalan-induced cytotoxicity in RPMI8322 melanoma cells (Ringborg et al., 1990). S-nitroso-compounds, such as S-nitroso-L-glutathione, inhibited the GST of rat liver by competing with GSH for its binding site at the reactive centre of the GST enzyme (Akman et al., 1988).

The role of GSH at the nuclear level is still unclear. Previous studies indicated that GSH reacts *in vitro* with monofunctional adducts of platinated DNA (Eastman, 1987). Such a reaction would prevent, and thereby decrease, the formation of more toxic bifunctional cisplatin-adducts and thus reduce the cytotoxicity of cisplatin. Data supporting this evidence emerged from a variety of studies. Experiments performed on a cisplatin-resistant mouse leukemia L1210 cell line indicated that such a resistance might be due in part to

enhanced quenching of monoadducts (Micetich et al., 1983) thus suggesting a critical role for GSH in preventing platinum monoadduct rearrangements. In a cisplatin-resistant hSCLC cell line characterised by a decrease in total DNA platination, a decrease in intra- and interstrand cross-links formation or an enhanced DNA repair capacity and no change in cellular platinum content were observed as compared to the parental cell line (Hospers et al. 1988, 1990a,b; Meijer et al., 1990a). GSH-depletion with BSO resulted in an increase in the amount of platinum bound to DNA as well as in the formation of Pt-GG adducts (Meijer et al., 1990a). These data would appear to suggest that high levels of GSH are responsible for the reduction of reactive platinum resulting in lower net platination and, consequently, in lower levels of intrastrand and interstrand cross-links (Hospers et al., 1990b; Meijer et al., 1990a).

The involvement of MTs in acquired resistance to cisplatin is rather controversial. MTs can be induced by heavy metals such as cadmium and zinc. Cadmium resistance induced *in vitro* in a human epithelial and a mouse fibroblast cell line, resulted in high levels of MTs and cross-resistance to cisplatin, In cytosols from cells resistant to this drug, 70% of the platinum appeared in the MTs fraction, whereas less than 5% was recovered in the corresponding fraction from non-resistant cells (Bakka et al., 1981). Induction of MTs by chronic culture in cadmium chloride and zinc chloride also led to cross-resistance with cisplatin in human ovarian carcinoma cells (Andrews et al., 1987). A 17% of the total cellular platinum in cadmium resistant cells was associated with the MTs fraction, against only 4% in the parent cells. It was

also demonstrated that cellular resistance to cisplatin is associated with an increase in MTs and their mRNA level in a panel of human cell lines, and that reversal of the cisplatin resistant phenotype is accompanied by a decrease in MTs content. Another study reported that the introduction of an eukaryotic expression vector encoding MT IIa (a human MT isoform) into mouse C217 cells conferred the cisplatin resistance phenotype to the cells (Kelley et al., 1988). Conversely, in a Chinese hamster ovarian cancer cell line resistant to cisplatin, no increase in MTs mRNA or MTs gene amplification was observed (Saburi et al., 1989). Moreover, results from other studies demonstrated that overexpression of MT mRNA could not be related to cisplatin-resistance in a panel of human ovarian cancer cell lines resistant to cisplatin as well as in a series of human ovarian cancer cells directly obtained from untreated patients and patients whose tumors were refractory to cisplatin-containing chemotherapy (Schilder et al.,1990). Although MT overexpression may be associated to cisplatin resistance induction, its causal role remains to be established.

1.3.3 Cellular DNA repair pathways and cellular resistance/tolerance to cisplatin-induced DNA damage

It has been widely accepted that genomic DNA is the primary target for cisplatin and its derivatives. Approximately 1% of the cellular platinum binds to DNA inducing covalent lesions such as monofunctional adducts and

bifunctional (interstrand, intrastrand and DNA-protein cross-links) adducts. Results from several studies illustrated the importance of induction and removal of the DNA lesions produced by cisplatin in determining cell sensitivity to this drug. It has been demonstrated that in human microcitoma cell lines resistant to cisplatin a very low level of cisplatin-DNA adducts (in particular interstrand cross-links) was present (Hospers et al., 1990b). Moreover, an increased extent of DNA-lesion repair was observed in a cisplatin-resistant human teratoma cell line with respect to the parental counterpart (Hill et al., 1990). Again, studies performed on human and rat ovarian cancer cell lines, revealed a greater induction of the unscheduled DNA synthesis in cisplatin-resistant cells than in sensitive cells.

The fact that a different cytotoxic activity of alkylating agents and platinum-based drugs was found in cell lines characterised by alterations in some of the most important DNA repair pathways suggested a strong correlation between DNA repair system functional status and cellular sensitivity to DNA damaging anticancer drugs.

Damaged DNA repair is a molecular process essential to the maintenance of life, evolved at an early time for the restoration of the original status of the genome (Hanawalt, 1995). Repair mechanisms contribute to the genetic stability maintenance and the physiological relevance of such pathways is evidenced by the fact that these systems operate in all organisms from bacteria to mammals and are surprisingly similar. Nevertheless, most DNA repair processes are error prone. The lack of a 100% efficiency in repair mechanisms

has contributed to the genetic diversity on earth and to the generation of variants in populations within species (Boulikas, 1992).

The repair of damaged DNA is an important molecular defence system against endogenous and exogenous events which, targeting genomic DNA, could be potentially mutagenic and lethal.

Endogenous DNA lesions can arise from:

- normal DNA metabolism processes such as replication and recombination which can produce both the formation of single-base mismatches or small insertions/deletions introduced by DNA polymerase and large insertions/deletions respectively;
- chemical reactions such as DNA hydrolysis, hydrolytic deamination of cytosine, 5-methylcytosine, adenine and guanine to uracil, thymine, hypoxanthine and xanthine respectively, depurination via hydrolytic breakage of the glycosyl bond linking DNA bases and sugar-phosphate backbone, hydroxyl (OH•) and superoxide (O_2•)-induced oxidation to produce oxidised DNA bases such as 8-oxoguanine/hydroxoguanine and 5,6-dihydroxy-dihydrothymine and, finally, methylation in which S-adenosylmethionine acts as methyl groups donor thus producing N^7- and O^6-methylguanine and N^3-methyladenine.

DNA damages can arise also from the action of exogenous factors including:

- ultraviolet light which is able to directly modify some DNA bases thus producing cyclobutane pyrimidine dimers (T<>T and C<>T), 6-4 photoproducts between two adjacent pyrimidines (T<>C), cytosine hydrate and thymine glycol;
- ionizing radiations which are able to interact both with the sugar-phosphate backbone and with single DNA bases thus producing single and double strand breaks;
- chemicals such as methylating agents, benzene, benzopyrene and anticancer drugs most of which produce carcinogen DNA damages (Karran and Bignami, 1994).

Such DNA lesions are all virtually repaired by the different DNA repair systems although the efficiency of each repair process depends on the chemistry of the lesion, the intrinsic cellular capacity for repair and the structure and dynamics of the chromatin (active transcription and replication) (Bohr, 1991). These considerations are also valid for lesions produced by anticancer drugs which are able to induce several different DNA lesions such as modified nucleotides, DNA cross-links and DNA single- or double-strand breaks.

DNA lesions induced by these drugs can be repaired in the following ways (Salles et al., 1995; van Vuuren, 1995; Wood, 1996).

Direct repair of the damaged base. This mechanism is used to repair minor damage, such as simple alkylation of bases caused by atmospheric methyl halides and the products of nitrate metabolism. The most important enzymes involved in this pathway are MGMT (O^6-methylguanine-DNA methyltransfrases; Wood, 1996) which are small proteins, highly conserved during evolution, that act by mobilising the methyl group on the O^6 position of guanine and sequestering it on a designed acceptor cysteine within the MGMT molecule itself. Transfer of the methyl group to the protein inactivates the MGMT. Thus the reaction is stoichiometric with each MGMT molecule able to act once. An important corollary of this is that the cell's capacity to repair O^6-methylguanine lesions is correlated strongly to the number of preexisting MGMT molecules.

Another important enzyme able to directly repair the damaged nucleotide is the photolyase enzyme which is involved in removal of cyclobutane pyrimidine dimers (photoproducts) (van Vuuren, 1995).

Multienzymatic DNA repair pathways. While direct repair is performed by only one enzyme, three different multienzyme systems are involved in the removal of damaged regions of DNA: Base Excision Repair (BER), Nucleotide Excision Repair (NER), Post-replication daughter strand repair, Mismatch Repair (MMR) and Recombinational repair.

BER is a "very short patch repair" able to remove simple types of lesion that result in relatively minor structural alterations of individual DNA bases

(produced by different chemical or physical agents: oxidative agents, X-rays or alkylating agents), such as N^3- and N^7-methyladenine, thymine glycols and G-T mispairs which are removed from DNA as free bases. This repair process is initiated by enzymes called DNA glycosylases that cleave the bond between the damaged base and the sugar-phosphate backbone leaving an apurinic/apyrimidinic site which is subsequently recognised and removed by an AP-endonuclease and a DNA-deoxyribose phophodiesterase. The gap in the DNA sequence is then filled by DNA polymerase β and a DNA ligase. (Sancar, 1995; Sobol et al., 1996).

The NER is a "short patch repair" system which recognises a wide variety of structurally unrelated DNA lesions, such as UV photoproducts and bulky adducts, including those produced on DNA by cisplatin. The NER process involves several steps: after recognition of the lesion, the damaged DNA strand is incised at both sides of the adduct. Next, the damaged base(s)-containing region is removed, the gap is filled by DNA reparative synthesis and finally the remaining nick is ligated (Sancar, 1996; Mu et al., 1996). NER may be coupled to transcription and acts in an ATP-dependent fashion.

If replication is blocked by a lesion, post-replication daughter strand repair can re-initiate downstream if the lesion is in the leading-transcribed strand. Replication can also restart 3' of the damage if the lesion is in the lagging strand. The single-stranded gaps, that are left in the newly synthesised DNA strand in this process, are repaired by recombinational strand exchange, using

the daughter strand as a template, allowing complete replication of the DNA (Kaufmann, 1989b).

MMR is also a post-replicational type of repair. This "long patch repair" pathway involves several steps: after damage recognition, the newly synthesised strand is cleaved, followed by degradation of the DNA region containing the mismatch. Finally, the gap is filled and ligated by DNA polymerase and ligase respectively (Kunkel, 1995).

The recombinational repair pathways, Non Homologous End Joining (NHEJ) and Homologous Recombination Repair (HRR), are known to be involved in the processing of very cytotoxic DNA lesions such as double-strand breaks (Takata et al., 2000) and DNA interstrand cross-links (Cole, 1973; Cole et al., 1976; Jachymczyk et al., 1981). Repair of DNA double-strand breaks by HRR requires the presence of homologous duplex DNA elsewhere in the genome, i.e., either a homologous chromosome or a sister chromatid. It commences by partial degradation of the double-strand break, leaving single-stranded ends, followed by protein binding to the free ends. Subsequently, strand exchange with duplex DNA is initiated (van Vuuren, 1995). NHEJ simply acts to process and ligate broken ends without a requirement for extensive homology (Takata et al., 2000).

There is a substantial overlap in the substrate specificity of these systems and it is important to underline that these DNA repair pathways do not act independently but are closely correlated since they are able to recognise similar

substrates, although with different affinity. For example, O^6-methylguanine adducts could be directly recognised and repaired by MGMT but it could also be recognised as O^6-meG-C or O^6-meG-T mismatch by the MMR system. Cisplatin induced intrastrand cross-links can be recognised by both MMR and NER. The NER pathway, in particular, is thought to remove virtually any lesion (Wood, 1996; Sancar, 1995). Repair of cisplatin-DNA adducts is believed to occur mainly by this pathway (Kunkel, 1995). Interstrand cross-links might also be repaired by the NER system. However, evidences emerged from several studies would suggest that the removal of such DNA lesions requires the participation of NER and recombinational repair proteins (Cole, 1973; Cole et al., 1976; Jachymczyk et al., 1981; Zamble and Lippard, 1995; Sancar, 1995; Wood, 1996).

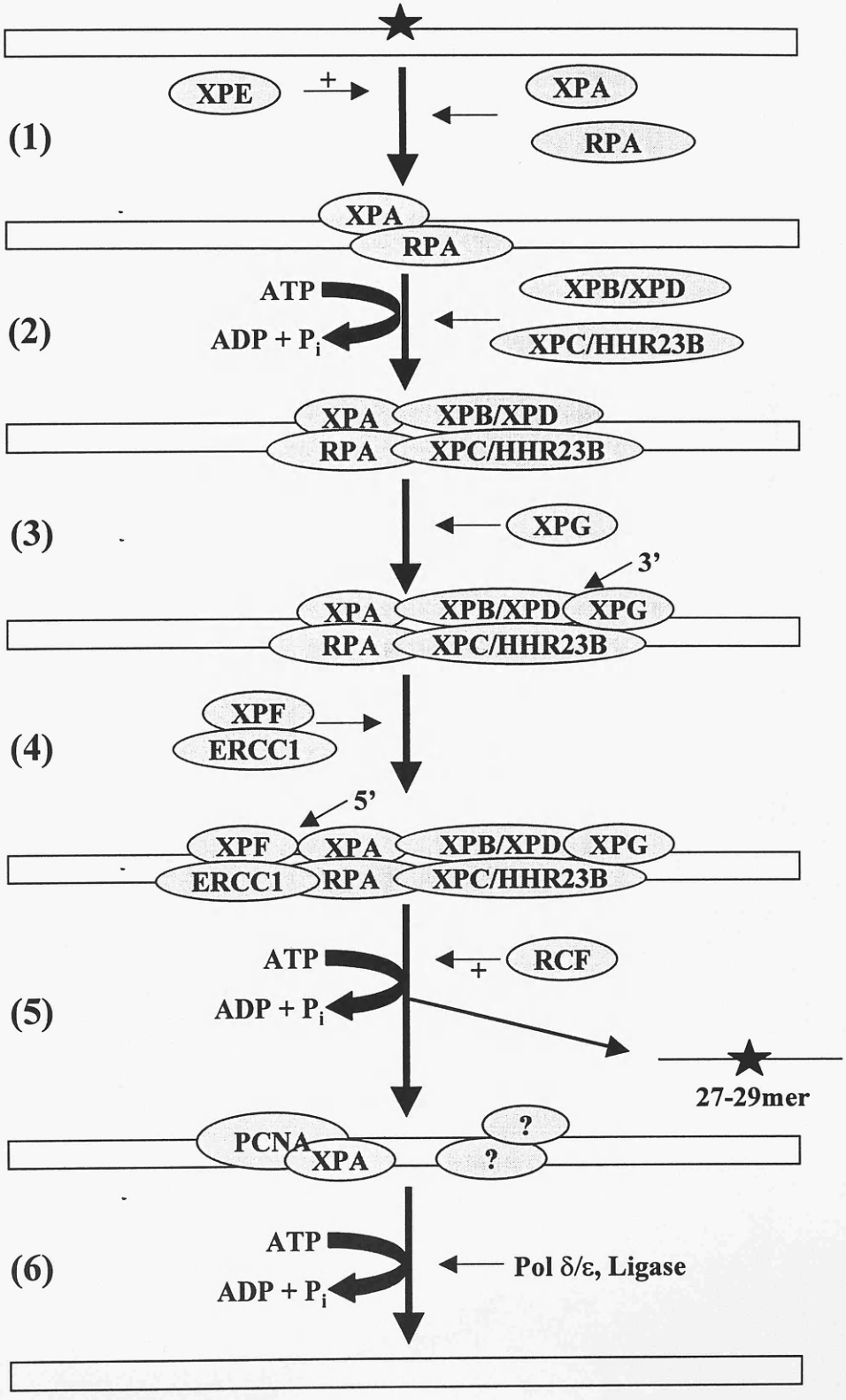
No involvement of the BER pathway in elimination of cisplatin-DNA lesions has been demonstrated. As regards MMR, very recent studies have demonstrated the ability of this system to recognise intrastrand cross-links (Yamada et al., 1997; Fink et al., 1998) and, in an attempt to repair these, MMR seems to be able to generate molecular signals triggering apoptosis.

Due to their great importance in determining cellular sensitivity to cisplatin and its derivatives NER, Recombinational Repair and MMR will be illustrated in detail.

1.3.3.1 Nucleotide excision repair (NER)

The NER system is one of the most important DNA repair mechanisms, because of its wide substrate range (Wood, 1996; Sancar, 1995). In humans, the NER pathway has been studied by means of three inherited disorders (Xeroderma pigmentosum (XP), Cockayne syndrome (CS) and TTD, a photosensitive form of trichotiodystrophy) in which a defective NER mechanism is involved. Genes encoding for proteins involved in NER have been named as the subclasses of XP in which they are lacking, whereas others have been designated ERCC genes (excision repair cross-complementing genes). Some independently identified XP proteins and ERCC gene products are identical (for example, ERCC2 is identical to XPD and ERCC3 is identical to XPB; O'Donovan and Wood, 1993). The NER reaction mechanism is represented in Figure 5. First, the damage is recognised by XPA-RPA (replication protein A, also referred to as 'human single-stranded binding protein' (HSSB) complex) (step 1). XPA contains a zinc finger and has a high affinity for damaged DNA. XPE is thought to play a role as a stimulatory or accessory factor for the binding of XPA-RPA to DNA. Next (step 2), the DNA is unwound by two subfactors of the general transcription factor TFIIH: XPB and XPD. These two factors exhibit helicase activities, each in opposite direction (XPB 3→5 and XPD 5→3). The XPC-HHR23B complex also binds to the damaged strand in this phase of the process and acts as a stabiliser for the unwound state of the DNA. Next (step 3 and 4), the dual incisions are made.

Figure 5. Model for nucleotide excision repair (NER) in humans. Damage (★) is recognised by the XPA-RPA complex, stimulated by XPE (Step 1). Subsequently, two subfactors of TFIIH, XPB and XPD, unwind the DNA after which the XPC-HR23B complex is recruited (Step 2). Next, XPG makes the 3' incision (Step 3) and XPF-ERCC1 makes the 5' incision (Step 4). The damaged part is released and the postincision complex is dissociated by RCF, leaving behind the gapped DNA protected by RPA, PCNA and other proteins (Step 5). Finally, the gap is filled and ligated (Step 6).


(1)
XPE
+
XPA
RPA
XPA
RPA
(2)
ATP
ADP + Pi
XPB/XPD
XPC/HHR23B
XPA
XPB/XPD
RPA
XPC/HHR23B
(3)
XPG
3'
XPA
XPB/XPD
XPG
RPA
XPC/HHR23B
(4)
XPF
ERCC1
5'
XPF
XPA
XPB/XPD
XPG
ERCC1
RPA
XPC/HHR23B
(5)
ATP
ADP + Pi
+
RCF
27-29mer
PCNA
XPA
?
?
(6)
ATP
ADP + Pi
Pol δ/ε, Ligase

-Figure 5-

First, XPG incises 3' of the lesion and remains bound to the damaged site. It plays a structural role in maintaining a stable complex and in recruiting XPF-ERCC1, which incises 5' of the lesion (Hoeijmakers and Bootsma, 1994). XPA also acts as an anchor to recruit XPF-ERCC1. The damaged part is now released as a 27-29 nucleotide long fragment (step 5) then the post incision complex is dissociated by a protein called RCF (replication factor C). Both the gapped DNA and the excised oligomer are complexed with proteins. Two of these proteins are proliferating cell nuclear antigen (PCNA) and RPA; the others have not yet been identified. Complexation is a necessary step in order to protect the excision gap from non-specific nucleases until repair synthesis take place. Repair is performed by DNA polymerases δ or ε. Finally, a ligase seals the remaining nicks (step 6).

It has been demonstrated that NER status can contribute to determine cellular sensitivity to different classes of DNA-damaging anticancer drugs such as cisplatin and minor groove-DNA alkylators Tallimustine and CC-1065 (Damia et al., 1996). Specifically, a role for enhanced DNA repair in cisplatin resistance has been observed frequently (Andrews and Howell, 1990; Calsou and Salles, 1993). In several resistant rodent cell lines, increased repair of cisplatin adducts was demonstrated (Eastman and Schulte, 1988; Sekya. et al., 1989; Sheibani et al., 1989). In one of the sensitive cell lines, the L1210 mouse leukaemia cell line, a mutation in the XPG gene was found and increased repair rates of DNA damage in a resistant L1210 subline could be attributed to the reversal of this mutation (Vilpo et al., 1995). The existence of such mutations

in other cell lines is not known. Increased repair of cisplatin-DNA adducts (measured by atomic absorption spectroscopy) has also been demonstrated in resistant human tumour cell lines (Ali-Osman et al., 1994; Bedford et al., 1988; Masuda et al., 1990; Parker et al., 1991). For example, the cisplatin resistant ovarian cancer cell line A2780cp70 was found to be about two-fold more efficient in repairing cisplatin adducts than the sensitive A2780 cells (Johnson et al., 1994; Masuda et al., 1990; Parker et al., 1991). A study on human bladder and testicular tumour cells showed that the less sensitive (RT112) was able to repair all platinum-DNA adducts whereas the five-fold more sensitive line (SUSA) was unable to remove the majority of DNA platination during the same period of post treatment (Bedford et al., 1988).

Increased repair of cisplatin-DNA lesions has also been demonstrated in patient derived cells. In one study, the rate of DNA repair in tumor cells from patients undergoing cisplatin treatment was quantified. A 2.8-fold increase in repair rate was found in tumour cells obtained from patients who underwent progression after cisplatin therapy, as compared to the level of repair in tumour cells obtained before treatment (Ali-Osman et al., 1994). Moreover, a study in tumour cells obtained from different individuals prior to treatment revealed that intrinsic DNA repair capacity varied as much as 10-fold (Jones et al., 1994). As a consequence, repair capacity in untreated tumours may be correlated to the clinical outcome of subsequent treatment, and information on this subject could be relevant for the clinical use of cisplatin.

Repair efficiencies of different types of cisplatin adducts vary considerably. In *in vitro* assays using mammalian cell-free extracts it has been demonstrated that the most abundant 1,2-d(GpG) intrastrand adduct is repaired poorly as compared to the other cisplatin DNA adducts (Szymkowski et al., 1992). In this system, the 1,2-d(ApG) and 1,3-d(GpG) intrastrand adducts were removed 30-50% more efficiently than the 1,2-d(GpG) intrastrand adduct (Szymkowski et al., 1992; Huang et al., 1994). The repair rates for interstrand cross-links and monofunctional adducts are difficult to measure. Since they are formed in very low amounts, highly sensitive assays are required. High disappearance rates of monofunctional adducts were reported in drug resistant cells but, since such lesions are potential precursors for bifunctional adducts, it is probable that this high rate of disappearance reflects adducts rearrangement rather than DNA repair (Bedford et al., 1988). In fact, when monofunctional platinum adducts (which were unable to rearrange to bifunctional adducts) were investigated in an *in vitro* assay using human cell-free extracts, no repair for these adducts was observed (Calsou et al., 1992). In an *in vitro* system using mammalian cell-free extracts, no repair of interstrand cross-links was detected, which indicated that repair of interstrand cross-links by the mammalian NER pathway is much less efficient than that of any of the intrastrand adducts (Szymkowski et al., 1992). In contrast, in other studies, performed with rat ovarian tumour cells and non-small cell lung cancer cell lines, interstrand cross-links removal was observed. In both cases, resistant sublines did not show increased repair of the interstrand cross-links (Bungo et al., 1990; Zeller et al., 1991). However, several other

groups reported an increased repair of interstrand cross-links in resistant tumour cell lines as compared to sensitive ones (Bedford et al., 1988; Hill et al., 1990; Calsou et al., 1992; Jones et al., 1991; Zhen et al., 1992; Johnson et al., 1994). The differences in rate of repair between the different cisplatin adducts may be due to specific distortions of the DNA helix induced by these adducts. It is believed that the repair proteins recognise the change in DNA conformation rather than the platinum compound itself (van Vuuren, 1995).

The NER mechanism involves several steps, each step requiring several proteins. It is not clear which step is rate limiting. In fact, both damage recognition and the incision step have been postulated to be rate-limiting in the NER process (Visse, 1994; Chu and Chang, 1990; Shivji et al., 1992). In order to determine the mechanisms responsible for the increase in NER activity in resistant tumour cells, the relative levels of proteins involved in NER have been measured (Tab. I).

The gene encoding for the general recognition factor XPA was found to be more actively transcribed in ovarian tumour cells from patients clinically resistant to platinum compounds, as compared to those obtained from patients clinically sensitive to these agents (Dabholkar et al., 1994). The levels of the other general recognition protein RPA have been investigated *in vitro*, in human ovarian cancer cell lines. Conversely, no overexpression of the other general recognition protein RPA, was observed in cisplatin-resistant sublines of human ovarian cancers as compared to parental cells (Bissett et al., 1993; Clugstone et al., 1992). Expression of the stimulatory factor XPE has also been

Table I. Proteins involved in nucleotide excision repair (NER).

Protein	General features	Overexpression in cisplatin-resistant cells
XPA	General DRP in NER	Yes
RPA/HSSB	General DRP in NER	No
XPE	DRP in NER with a higher affinity for u.v. damage than for cisplatin adducts	Yes
ERCC1	Protein involved in making the 5'incision in NER	Yes
ERCC2/XPD	Helicase in NER	No
ERCC3/XPD	Helicase in NER	No

-Table I-

studied in several human tumour cell lines. Studies performed with HeLa cell extracts showed that two nuclear factors that recognise UV-modified DNA were overexpressed in the cisplatin-resistant sublines (Chao et al., 1991a). One of these factors is believed to be XPE and the other has not been identified. These cisplatin-resistant HeLa cells showed cross-resistance to UV-induced damage supporting the hypothesis that XPE is involved in repair of cisplatin adducts as well as of UV damage. Another study from the same research group demonstrated that these UV-damage recognising factors were inducible by cisplatin (Chao et al., 1991b). Moreover, in human tumour cell lines of various origin a 2.7 to 4.3-fold expression of XPE was found in cisplatin-resistant cells with respect to their cisplatin-sensitive parental cells (Chu and Chang, 1990). It was also demonstrated that these cisplatin resistant cells had acquired an increased DNA repair activity. In the cisplatin-resistant cell lines described above, the levels of two of the three known DRPs (Damage Recognition Proteins) involved in NER were increased and, therefore, it is reasonable to believe that cisplatin can influence the expression of these proteins. High levels of these DRPs may lead to a more efficient repair of the cisplatin-DNA adducts and hence induction of cisplatin resistance. Also, it seems likely that cells that overexpress XPA and XPE proteins may acquire cross-resistance to DNA active drugs other than cisplatin, since these factors are capable of recognising multiple forms of damaged DNA (Chu and Chang, 1990).

ERCC1, which is involved in making the 5' incision in the NER pathway, is considered the most important marker for NER activity in several studies

(Reed, 1996a,b). In fact, increased levels of ERCC1 were observed in tumour cells from patients with ovarian cancer resistant to cisplatin chemotherapy (Dabholkar et al., 1994; Dabholkar et al., 1992). In an *in vitro* study, pronounced differences in the basal levels of ERCC1 were observed between sensitive and resistant ovarian cancer cells, with the resistant cell line showing a 30- to 50-fold higher level of ERCC1 than the sensitive one.

In vitro studies carried out on NER deficient-Chinese hamster ovarian carcinoma cells lacking functional ERCC1, 2, 3, 4, 5 or 6 genes, confirmed that ERCC1 and ERCC4 genes play a fundamental role in the repair of cisplatin and melphalan-induced DNA lesions since cells lacking such proteins were hypersensitive to these drugs (Damia et al., 1996). These findings were supported by data emerging from experiments in which transfection of ERCC1 mutant cells with an expression vector carrying the wild type ERCC1 gene rendered them more resistant to both cisplatin and melphalan (Bramson and Panasci, 1993).

The importance of a functional ERCC1 protein was also demonstrated in a study performed on human ovarian cancer and T cells in which it was shown that ERCC1 may exist in at least two forms: a full length form and an alternatively spliced form that lacks exon VIII (van Duin et al., 1986; Dabholkar et al., 1994) and codes for a functionally inactive ERCC1 protein. It seems probable that the portion of the protein codified by the exon VIII is responsible for making the 5' incision into cellular DNA. In cisplatin resistant cells, the molar ratio between the alternative spliced form and the full length

form of ERCC1 was found to be lower than that of cisplatin-sensitive cells, thus indicating a possible correlation between the level of alternative splicing of ERCC1 and cisplatin-DNA lesion repair activity of the NER.

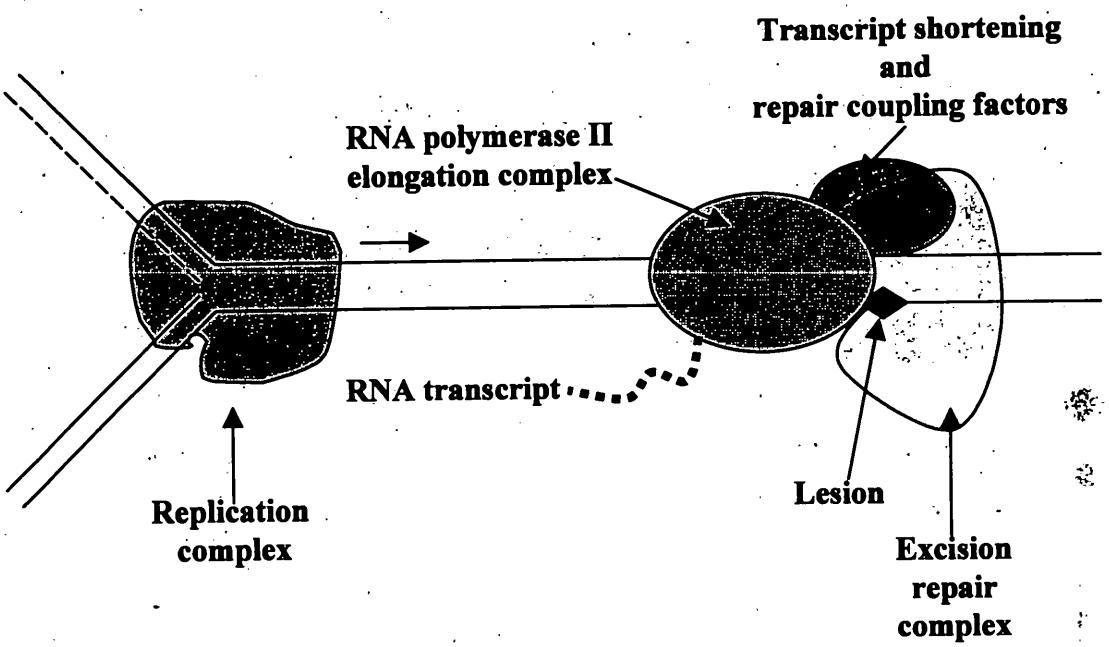
These data altogether indicate that clinical resistance to cisplatin seems to be associated with an increased expression of ERCC1 protein and/or a higher 'alternative spliced form/full length form' molar ratio of the ERCC1 mRNA in ovarian cancer patients (Dabholkar et al., 1994). Moreover, in a study performed on parental Chinese hamster ovarian cancer cells, it was found that overexpression of the ERCC1 resulted in a hypersensitivity to cisplatin and melphalan. It was suggested that the overexpression of ERCC1 protein would prevent the dissociation from DNA of the incision complex, thus inhibiting repair, or alternatively lead to more incisions than other NER proteins could repair (Bramson and Panasci, 1993).

As regards the two helicases, XPD (ERCC2) and XPB (ERCC3), results obtained from studies carried out on ovarian cancer and testicular tumour cells showed an upregulation of these in cisplatin-resistant cells as compared to sensitive cells (Dabholkar et al., 1992; Taverna et al., 1994).

Increasing evidence has become available regarding the presence of a considerable intragenomic heterogeneity of the DNA repair processes. Since the genes only constitute a small fraction of the overall genome (<1%), any changes in gene-specific repair would not be detected using traditional assays for the overall genome (Bohr, 1991). DNA repair appears to take place

preferentially on the transcribed strand of expressed genes (Sancar A., 1995; Friedberg, 1996; Hanawalt, 1994; Bohr, 1991). Moreover, preferential repair is confined to genes which are transcribed by RNA polymerase II (Sancar, 1995). The current hypothesis for the reaction mechanisms of transcription-coupled repair has been described (Friedberg, 1996): Briefly, when RNA polymerase II encounters a lesion during transcription, it stalls. The stalled complex (Fig. 6) is recognised by transcription proteins (probably TFIIS), by proteins CSA and CSB (McLaughlin et al., 1993), and by the product of a gene named Mfd (i.e. transcription repair coupling factor TRCF). TRCF is believed to bind DNA specifically at a site where RNA polymerase II is stalled by interacting with DNA-RNA polymerase II complex (Friedberg, 1996). Subsequently, RNA polymerase II backs up or is removed, and the recognition protein-complex recruits XPA and TFIIH to the lesion site that is now accessible. The NER complex (XPG, XPF-ERCC1) then assembles at this site, and repair synthesis takes place, restoring a lesion-free duplex that can now serve as a template for transcription as well as in NER. Two types of TFIIH complex have been isolated from yeast and are referred to as the TFIIH holoenzyme and the nucleotide excision repairosome (Drapkin et al., 1994; Haldar et al., 1994). These complexes share some components but also have some different, unique features. The relationship between these two forms is still unclear. However, it is likely that a fraction of the NER system is specifically committed to, and active on, the transcribed strand of active sequences in the genome. (Sullivan, 1995).

Figure 6. Model for transcription-coupled DNA repair in mammalian cells. Arrested RNA polymerase II undergoes a conformational change to initiate transcript shortening and reannealing of DNA strands at the lesion site. The coupling factor (or factors recruits TFIIH and the additional elements required for incisions, excision and repair synthesis. After repair, transcription resumes, thereby completing the RNA transcript in progress and facilitating the passage of an advancing replication fork.


Transcript shortening
and
repair coupling factors
RNA polymerase II
elongation complex
RNA transcript
Replication
complex
Lesion
Excision
repair
complex

-Figure 6-

Experimental research into repair of cisplatin adducts demonstrated that there is a higher repair rate in the actively transcribed genes than in the silent genes (May et al., 1993) or in the non-coding regions of the DNA (Zhen et al., 1992; Johnson et al., 1994). Moreover, higher repair rates of interstrand cross-links on active genes were detected in resistant rather than in sensitive human ovarian cancer cell lines (Johnson et al., 1994). For cisplatin-induced intrastrand cross-links, the difference was much smaller (Zhen et al., 1992). Intrastrand cross-links are preferentially removed from actively transcribed regions with strand specificity.

Repair of cisplatin DNA adducts as measured by atomic absorption spectrometry occurs in a biphasic manner: a rapid phase for the first 6 hours after drug incubation followed by a slower period (Johnson et al., 1994; Eastman and Schulte, 1988; Parker et al., 1991; Calsou et al., 1992; Bungo et al., 1990; Jones et al., 1991; May et al., 1993). Separation between the two phases is more clear in resistant than in sensitive cells, although this may be due to the relatively low levels of repair observed in the sensitive cell lines. (Johnson et al., 1994; Eastman and Schulte, 1988). As regards interstrand cross-link removal, the biphasic repair mode is less obvious as compared to overall platinum removal (Calsou et al., 1992). A probable explanation is that the rate of formation for interstrand cross-links is slower than that of other platinum adducts, as the interstrand cross-links are formed in two steps (monofunctional binding and then rearrangement to the interstrand cross-links). Intrastrand adducts are also formed in two steps, but at a greater pace than the

interstrand cross-links. When formation and removal are simultaneously in action, it is difficult to determine the relative contribution of each to the interstrand cross-links concentration at a given time point. When studying the repair kinetics for other DNA-damaging agents, such as UV, photoproducts and alkylating agents, a similar biphasic repair mode is obtained (May et al., 1993), implicating that initial fast DNA repair followed by slow DNA repair might be universal for NER and not specific for platinum adducts removal. The biphasic mode of repair probably indicates that NER is more active directly following induction of DNA damage. It is conceivable that the transcription-coupled repair process is faster than overall genomic repair.

1.3.3.2 Recombinational Repair

Recombinational repair systems are involved in the processing of DNA double-strand break lesions (Sweigert and Carrol, 1990), a particular class of DNA damages which can occur during DNA replication and which can be also generated after cell exposure to ionising radiation (Takata et al., 2000). It has been extensively demonstrated that the failure to repair these lesions is responsible for cell-killing induced by ionising radiation since the persistence of double-strand breaks on genomic DNA induces the formation of chromosome aberration preventing successful cell division at the next mitosis (Price, 1993).

Since double-strand breaks are so deleterious to the cells, it is not surprising that there are two repair pathways able to process these DNA lesions: Non Homologous End Joining (NHEJ) and Homologous Recombination Repair (HRR). The main difference between these two recombinational repair pathways is that HRR is able to process DNA double-strand breaks only in the presence of homologous duplex DNA in the genome (either a homologous chromosome or, more likely, a sister chromatid) whereas NHEJ is able to process these lesions without a requirement for extensive homology (Takata et al., 2000).

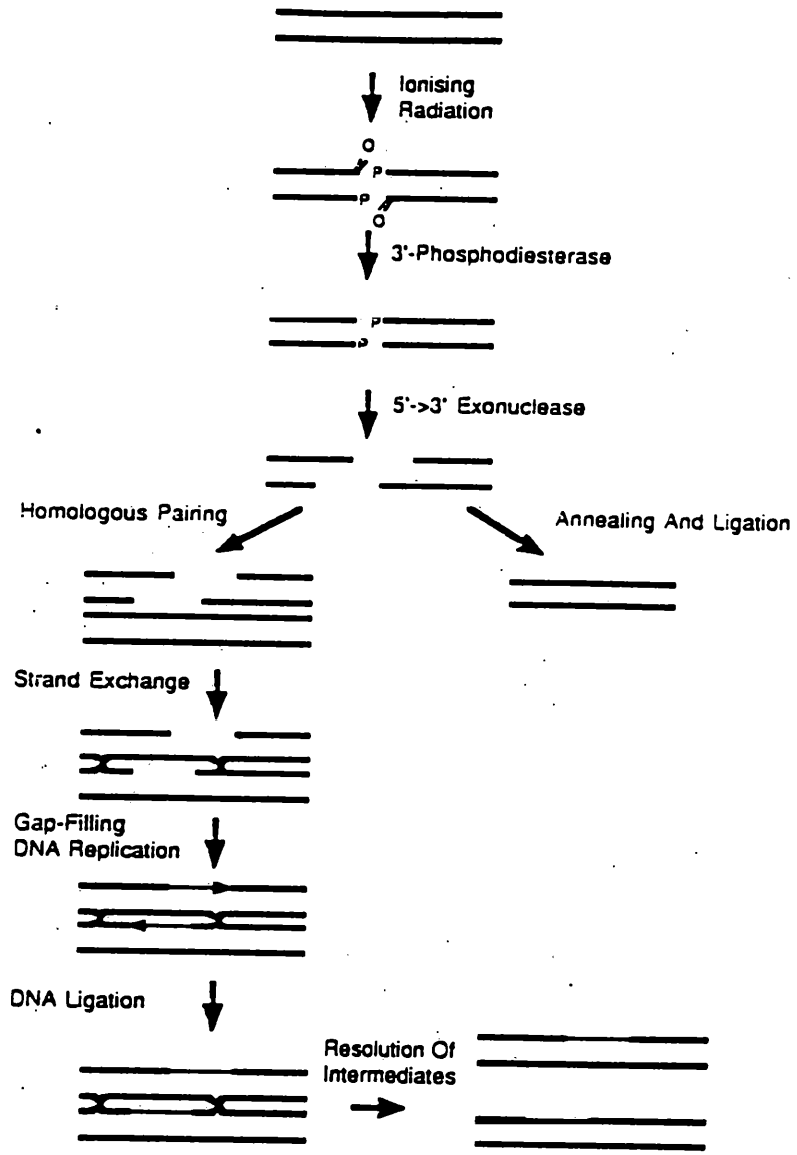
Results from several studies indicate that these two pathways are conserved from the bacteria to yeast (Shinoara et al., 1992; Aboussehkra et al., 1992; Basile et al., 1992) and that similar mechanisms persist from yeast to humans (Boulton et al., 1996; Siede et al., 1996; Thompson and Schild, 1999). Specifically, it has been found that while HRR is the primary mechanism of double-strand breaks repair in yeast, vertebrate cells use both NHEJ and HRR pathways extensively (Liang et al., 1998, Johnson et al., 1999; Lin et al al., 1999; Pierce et al., 1999).

Direct evidence for the repair of double-strand breaks induced by ionising radiation by the recombinational repair pathways was firstly obtained by microinjecting X-irradiated plasmid DNA into oocyte nuclei from Xenopus Laevis and was subsequently confirmed by experiments performed *in vivo* with the SCID mouse mutant which is characterised by a severe immunodeficiency due to a defect in V-(D)-J rejoining, a site-specific

recombinational event responsible for the huge diversity of DNA sequences in T cell receptor and immunoglobulin genes. The mice themselves as well as cell lines derived from them have been shown to be hypersensitive to ionising radiation (Fulop and Phillips, 1990; Hendrickson et al., 1991) probably due to a failure to repair double-strand breaks (Biedermann et al., 1991). It has been postulated that the animals lack an activity involved in an early stage of recombination which is necessary for both repair and V-(D)-J rejoining pathways (Hendrickson et al., 1991).

As mentioned above, two models of recombinational repair of double-strand breaks have been proposed: the single-strand annealing model (NHEJ; Lin et al., 1984) and the double-strand break repair model (HRR; Szostak et al., 1983) (Fig. 7). The NHEJ occurs at direct repeats. The DNA termini at the double-strand break are digested by the 5'->3' exonuclease until complementary sequences are exposed, when annealing and ligation occur. This provides a rapid means of repairing this form of damage, but deletions are inevitable. In the alternative method, the HRR, after that DNA termini at the double-strand breaks are digested by the 5'->3' exonuclease, an homologous pairing occurs between the segment of DNA containing the damage and the duplicate sequence from the second copy of this stretch of nucleic acid. Strand exchange, and possibly, branch migration, follow, and gap-filling DNA repair replication precedes resolution ot the recombination intermediates. This model implies that the nucleic acid at the site of damage is conserved without loss of genetic information. Direct repeats, which are a prerequisite for the NHEJ system, are

Figure 7. A model for the repair of ionising radiation-induced double-strand breaks. The altered residues at the 3'-termini are first excised by a 3'-phosphodiesterase. Thereafter, the double-strand gap is extended on both strands by a Mg^{2+}-dependent 5'→3' exonuclease. If direct repeats are present at the site of the DSB, then repair may occur by single-strand annealing of complementary sequences with deletion of the exonucleolytically-digested regions around the strand break. In stretches of DNA lacking direct repeats, including most of those which encode proteins, correction of the damage is carried out by the double-strand break repair pathway. Homologous sequences from the two chromosomal copies of the gene are paired, and strand transfer, DNA replication, DNA ligation and resolution of intermediates follow.


Ionising
Radiation
3'-Phosphodiesterase
5'->3' Exonuclease
Homologous Pairing
Annealing And Ligation
Strand Exchange
Gap-Filling
DNA Replication
DNA Ligation
Resolution Of
Intermediates

-Figure 7-

more likely to occur between protein-coding sequences, and these two mechanisms might be complementary, allowing the possibility to perform rapid repair of less important sections of the genome, and a slower but more accurate repair of segments containing key coding sequences.

The recombinational repair of double-strand breaks was firstly extensively elucidated in Escherichia coli (West, 1992) and then in eukaryotic systems, such as RAD52 epistasis group of Saccharomyces cerevisiae and mammalian cells. The analysis of radiosensitive yeast mutants has revealed a number of genes involved in HRR, which comprise the RAD52 epistasis group (Baumann and West, 1998; Kanaar et al., 1998; Shinohara and Ogawa, 1995). Among these, the structure and function of Rad51 have been conserved to a remarkable degree among all eukaryotes. Rad51 is structurally and functionally related to the E. coli recombination protein RecA (the agent responsible for catalysing homologous pairing and strand exchange in the bacterial HRR) (Kowalczykowski, 1994). The functional forms of both RecA and Rad51 are multimeric helical nucleoprotein filaments that form on single-stranded DNA ends produced at double-strand breaks (Ogawa et al., 1993). These filaments are involved in the search for homologous sequence, DNA pairing and strand exchange. As mentioned above, recombination intermediates produced in this way are then processed further in reactions that involve DNA synthesis, branch migration, resolution of Holliday junctions and ligation. The conservation of the RAD52 epistasis group genes from yeast to vertebrate cells suggests that the basic mechanism of HRR is maintained during evolution. However, while

S. cerevisiae RAD51 mutants are viable, RAD51 deficiency in vertebrate cells causes rapid chromosomal aberrations and cell death (Sonoda et al., 1998). One possible explanation for this lethality is that the larger size of the vertebrate genome requires more HRR activity for chromosome stability (Haber, 1999; Sonoda et al., 1998). RAD51 paralogs (genes related by duplication within a single genome) have been identified in many eukaryotes and constitute the Rad51-related gene family (Tacker, 1999; Thempson and Schild, 1999). Four proteins, Rad51, Rad55, Rad57 and Dmc1, constitute the complete set of RecA relatives in S. cerevisiae (Takata et al., 2000), whereas seven members of the Rad51 protein family have been identified, thus far, in mammals. In human cells, Rad51, Dmc1, XRCC2, XRCC3, Rad51B (also called Rad51L1/hRec2), Rad51C (also called Rad51L2) and Rad51C (also called Rad51L3) are highly conserved (Takata et al., 2000). While human Dmc1 is about 50% identical to human Rad51, the other human Rad51 paralogs are 20% to 30% identical to human Rad51. These paralogs are less than 30% identical to each other and to yeast Rad55 and Rad57 (Thacker, 1999). Overexpression of Rad51 in yeast partially suppresses the DNA repair defects of rad55 and rad57 mutant strains (Hays et al., 1995; Johnson and Symington, 1995), implying that Rad55 and Rad57 may functionally cooperate with Rad51. This idea is supported by physical interactions between Rad51 and Rad55 and between Rad55 and Rad57 (Hays et al., 1995; Johnson and Symington, 1995; Sung, 1997). Similarly, physical interactions occur between human Rad51 and XRCC3, XRCC3 and Rad51C, and between Rad51B and Rad51C (Dosanjh et al., 1998). These

findings suggest that Rad51 paralogs may function as Rad51 accessory factors, analogous to yeast Rad55 and Rad57.

It has been extensively demonstrated that germ line mutations in BRCA1 and BRCA2 genes result in a marked increase in the risk of early onset breast and ovarian cancers (Futreal et al., 1994; Friedman et al., 1994; Castilla et al., 1994; Wooster et al., 1995; Tavtigian et al., 1996). These two genes appear to have multiple functions including roles in transcriptional regulation (Chapman and Verma, 1996; Monteiro et al., 1996; Milner et al., 1997; Irminger-Finger et al., 1999) and cell cycle checkpoints control (Larson et al., 1997; Hsu and White, 1998; Patel et al., 1998). BRCA1 and BRCA2 have transcription activation functions (Chapman and Verma, 1996; Monteiro et al., 1996; Milner et al., 1997); BRCA1 co-activates transcription with p53 (Ouchi et al., 1998; Zhang et al., 1998). Very recent findings suggested that products of the BRCA1 and BRCA2 genes also participate in HRR by directly interacting with the recombinational repair protein Rad51 (Moynahan et al., 1999; Snouwaert et al., 1999) thus confirming results from previous studies showing that BRCA1 and BRCA2 mutants are phenotypically similar to rad51 mutants (Scully et al., 1997; Sharan et al., 1997; Abbott et al., 1998).

In addition to contributing to HRR of double-strand breaks on DNA, BRCA1 has also been involved in other DNA repair pathways like transcription-coupled base-excision repair of oxidative DNA damage (Gowen et al., 1998; Abbott et al., 1999). Furthermore, a recent study reported biochemical interactions between BRCA1 and proteins required for DNA-end joining,

nucleotide mismatch repair, DNA replication, and signal transduction in response to damage (Wang et al., 2000). This study also identified interactions between Brca1 and other proteins thought to be involved in recombinational repair. Although these data altogether raise the possibility the BRCA1 contributes to multiple cellular DNA damage responses, the specific mechanisms through which BRCA1 contributes to these processes remain to be determined.

Results from recent studies carried out in mouse ES cells indicated that BRCA1 is required for the formation of subnuclear Rad51 complexes in response to cellular damage by ionising radiation and cisplatin, thus suggesting that cellular sensitivity to DNA lesions generated by chemical cross-linking agents is mediated by the HRR. Accordingly, cells lacking normal BRCA1 or Rad51 functions are more sensitive to ionising radiation (Gowen et al., 1998) and cross-linking agents (Husain et al., 1998; Bhattacharyya et al., 2000) compared with normal cells.

Bacterial and yeast studies demonstrated that repair of interstrand cross-links generated on DNA by damaging agents such as cisplatin, mitomycin C, chloronitrosoureas and nitrogen mustards requires both participation of NER and recombinational repair proteins (Cole, 1973; Cole et al., 1976; Jachmczyk et al., 1981). In fact, yeast mutants defective in NER or HRR DNA repair systems show increased sensitivity to cisplatin, indicating that these repair pathways are involved in removal of the DNA lesions caused by this agent (Hartwell et al., 1997). It has been postulated that NER proteins are responsible

for lesion recognition and for single strand incision and/or double-strand breaks formation at the sites of damage whereas recombinational repair proteins are responsible for repairing the intermediates formed by NER proteins acting on interstrand cross-links. The intermediates acted on by recombinational repair proteins may include double-strand breaks formed by incision of both strands at the lesion, daughter strand gaps caused when replicative polymerases are blocked by lesions, or DNA ends formed when polymerases encounter single strand incisions. In the first case, recombinational repair can be employed to accurately 'heal' the double-strand break using a homologous duplex as a donor of sequence information; in the latter two cases, recombinational repair can be used to accurately restore a functional replication fork. Recombinational repair is also important for restoring replication forks when unrepaired intrastrand cross-links are encountered by DNA polymerases (Kadyk and Hartwell, 1992, 1993). Results from several studies indicated that certain DNA intrastrand cross-links generated by cisplatin and other damaging agents such as mitomycin C, chloronitrosoureas and nitrogen mustards are refractory to be removed via NER and translesion synthesis pathways (Hughes et al., 1992; Huang et al., 1994; Hoffmann et al., 1997). It has been hypothesised that this refractoriness likely results from the binding of high mobility group proteins to the adducts (Hughes et al., 1992; Huang et al., 1994; Hoffmann et al., 1997).

1.3.3.3 Mismatch repair (MMR)

Maintenance of genomic stability requires the proper functioning of DNA replication, repair, and recombination processes. Among these, mismatch repair (MMR) plays a prominent role in the correction of biosynthetic mistakes made during replication from newly synthesised strands that escape DNA polymerase proofreading (Modrich, 1994). In humans the MMR can repair modified bases, mispaired bases (for example T opposite G) or the addition of extra nucleotides up to a number of four, resulting in unpaired bases (Modrich, 1994; Kunkel, 1995). Heteroduplexes containing more than four unpaired bases are not efficiently corrected by the MMR system (Kunkel, 1995).

MMR was originally described in bacteria that are able to repair mispair-containing bacteriophages (Wildenberg and Meselson, 1975). In the bacterial system, the most important components are MutS, MutL and MutH gene products, named after their corresponding bacterial mutator strains. The MutS protein initially recognises and binds to mismatched DNA (Su and Modrich, 1986). After this, MutH and MutL form a complex with MutS to carry out excision repair; MutH has endonuclease activity, but the specific activity of MutL has not been identified. The important role played by the MMR proteins is emphasised by the fact that they are highly conserved from bacteria to yeast to mammals. Biochemical and genetic studies in human cells have defined five genes whose products play a key role in MMR including hMSH2 (Fishel et al., 1993; Leach. et al., 1993), hMSH3 (Fujii and Shimada, 1989; Risinger et al.,

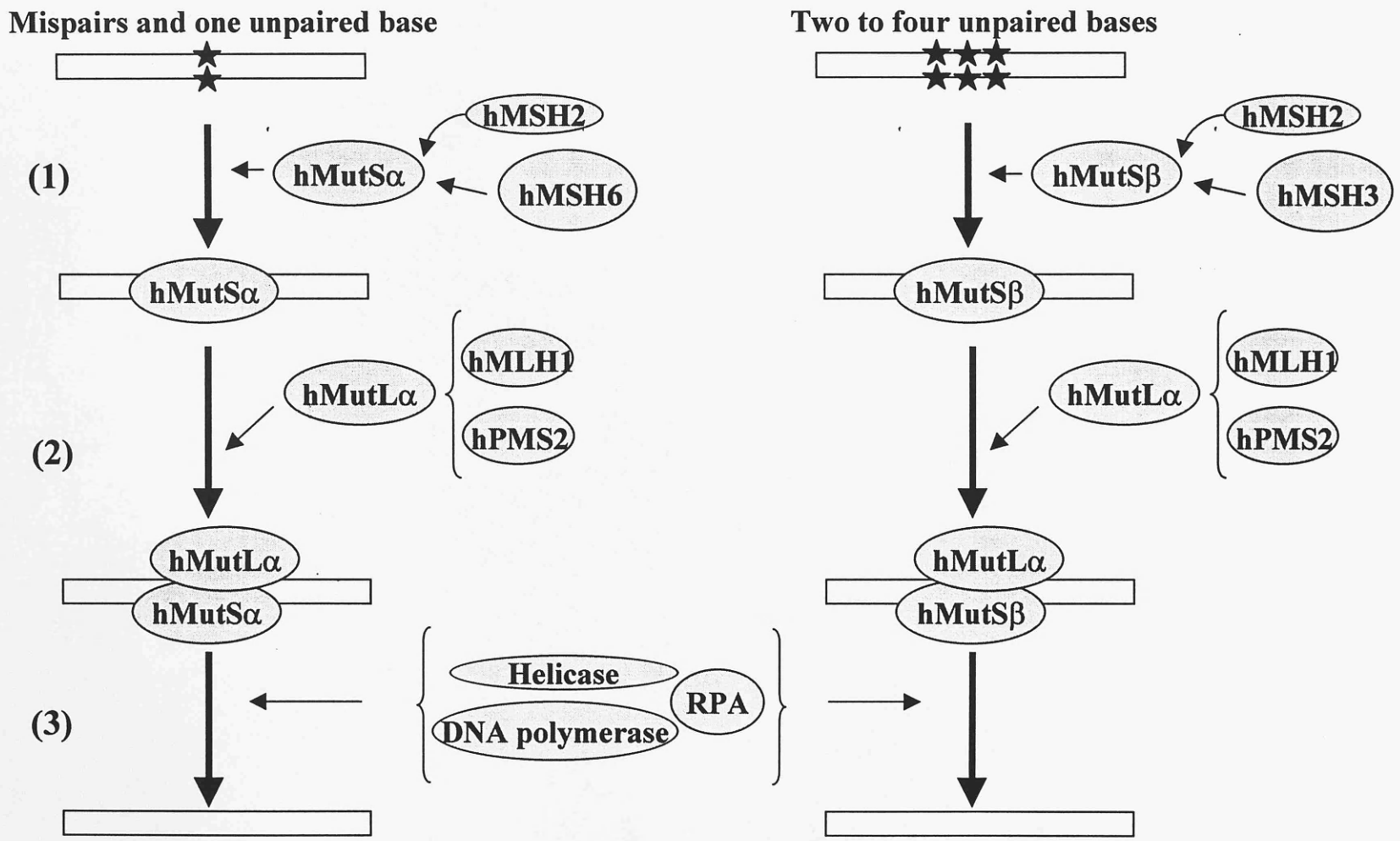
1996) and hMSH6 (also called GTBP or p160; Palombo et al., 1995; Drummond et al., 1995), which are homologues of the bacterial MutS protein, and hMLH1 (Papadopoulos et al., 1994; Bronner et al., 1994) and hPMS2 (Nicolaides et al., 1994), which are homologues of the bacterial MutL protein (Tab. II). A sixth human gene, hPMS1, has also been suggested to be important for MMR, although biochemical studies supporting its involvement in this repair process are not yet available (Kolodner, 1996). Both the bacterial and the eukaryotic systems direct the repair to the newly replicated DNA strand, require multiple components and can cut the strand to be repaired either upstream or downstream of the mismatch. How these recognition proteins discriminate between the newly synthesised strand and the mother strand in humans is still unclear. In bacteria, the newly synthesised strand is recognised by the lower level of methylation (Modrich, 1994). This hypomethylation probably also plays a role in the process of recognition in humans. hMSH2, either by itself (Fishel et al., 1994) or when dimerised with hMSH6 (Palombo et al., 1995) or hMSH3 (Acharya et al., 1996) binds small DNA mismatches (Fig. 8). The human MutL homologues, hMLH1 and hPMS2, form an heterodimer (called hMutLα; Li and Modrich, 1995) and join the complex after the initial binding by either hMSH2-hMSH6 (hMutSα heterodimer) or hMSH2-hMSH3 (hMutSβ heterodimer). Analysis of the mismatched nucleotide-binding specificity of the hMutSα and β protein complexes showed that they have overlapping but not identical binding specificity (Acharya et al., 1996). The human MMR pathway (Fig. 8) starts by the protein hMSH2 or MutSα (a complex composed of

Table II. Human homologues of bacterial MutS and MutL mismatch repair (MMR) proteins.

Bacterial MMR	Human Homologue	Size (KDa)	Chromosomal localisation
MutS	hMSH2	105	2p22-21
	hMSH3	127	5q11-13
	hMSH6	153	2p16-15
MutL	hMLH1	86	3p23-21
	hPMS1	106	2q31-33
	hPMS2	96	7p22

-Table II-

Figure 8. Model for mismatch repair (MMR) in humans. Damage (★) is recognised by hMSH2 and hMSH6 or hMSH3 (Step 1). Subsequently, hMLH1, hPMS1 and hPMS2 are recruited (Step 2). Then, the DNA is unwound by a helicase (Step 3), and a DNA polymerase repairr the damage with the aid of RPA.


Mispairs and one unpaired base
Two to four unpaired bases
hMSH2
hMutSα
hMSH6
hMutSβ
hMSH3
(1)
hMutSα
hMutSβ
hMutLα
hMLH1
hPMS2
(2)
hMutLα
hMutSα
hMutSβ
Helicase
RPA
DNA polymerase
(3)

-Figure 8-

hMSH2 and a protein named GTBP or p160) that recognises the mispaired bases (step 1). Which of the two proteins is active depends on the number of mispaired bases. Other recognition proteins, such as hMSH3, might function in this step of the MMR pathway. Following recognition, hPMS1, hPMS2 and hMLH1 interact with the DNA-MutSα binary complex (step 2). The exact role of these proteins is not known, but mutations in the encoding genes are associated with hereditary non-polyposis colorectal cancer (HNPCC), a disease characterised by a defective MMR system (Modrich, 1994; Chung and Rustgi, 1995). From this point on, many enzymes involved have not been characterised yet, but subsequent steps would include unwinding of the DNA by a helicase (step 3) and excision of the mismatched base containing DNA region by a bi-directional exonuclease. An as yet unidentified DNA polymerase fills the gap, possibly with the aid of RPA. Finally, the DNA is sealed by a ligase (Kunkel 1995; Chung. and Rustgi, 1995). The process is probably more complicated, as recently several other components required for MMR have been identified (Kolodner, 1996).

Loss of MMR causes destabilisation of the genome and results in high mutation rates, particularly in microsatellite sequences in both the non-coding (Aaltonen et al., 1993; Thibodeau et al., 1993) and coding portion of the genome. Such sequences are found in coding regions of HPRT (Bhattacharyya et al., 1994), APRT (Hess et al., 1994), APC (Huang J et al., 1996), type II TGF-β receptor (Markowitz et al., 1995), CHK-1 (Codegoni et al., 1999; Bertoni et al., 1999), E2F-4 (Souza et al., 1997), ICE (Souza et al., 1996)

hMSH3 (Yamamoto et al., 1998), hMSH6 (Yamamoto et al., 1998) and Bax (Rampino et al., 1997) genes, and mutation rates are increased at these loci in MMR-deficient cells. The majority of hereditary non polyposis colon cancers are due to defects in either MLH1 or MSH2 (Papadopoulos et al., 1994; Bronner et al., 1994; Hemminki et al., 1994; Liu B et al., 1996). Defects in PMS1 or PMS2 are less frequent (Nicolaides et al., 1994; Hemminki et al., 1994; Liu B et al., 1996). Although the MMR system seems to be normal in the heterozygote cells containing a single functional gene copy, during carcinogenesis, the remaining wild-type allele is somatically mutated, resulting in the complete loss of MMR function in the tumour (Leach et al., 1993). In addition to hereditary nonpolyposis colon cancer, loss of MMR occurs frequently in many types of sporadic cancers as well, including endometrial, small and non-small cell lung, pancreatic, gastric, ovarian, cervix, and breast cancer (Risinger et al., 1993; Merlo et al., 1994; Shridhar et al., 1994; Han et al., 1993; Wooster R. et al., 1994). Mice that are deficient in MLH1, MSH2 or PMS2 have microsatellite instability in many tissues and a predisposition to form tumours, particularly lymphoma (Baker et al., 1996; de Wind et al., 1995; Reitmar et al., 1995; Baker et al., 1995).

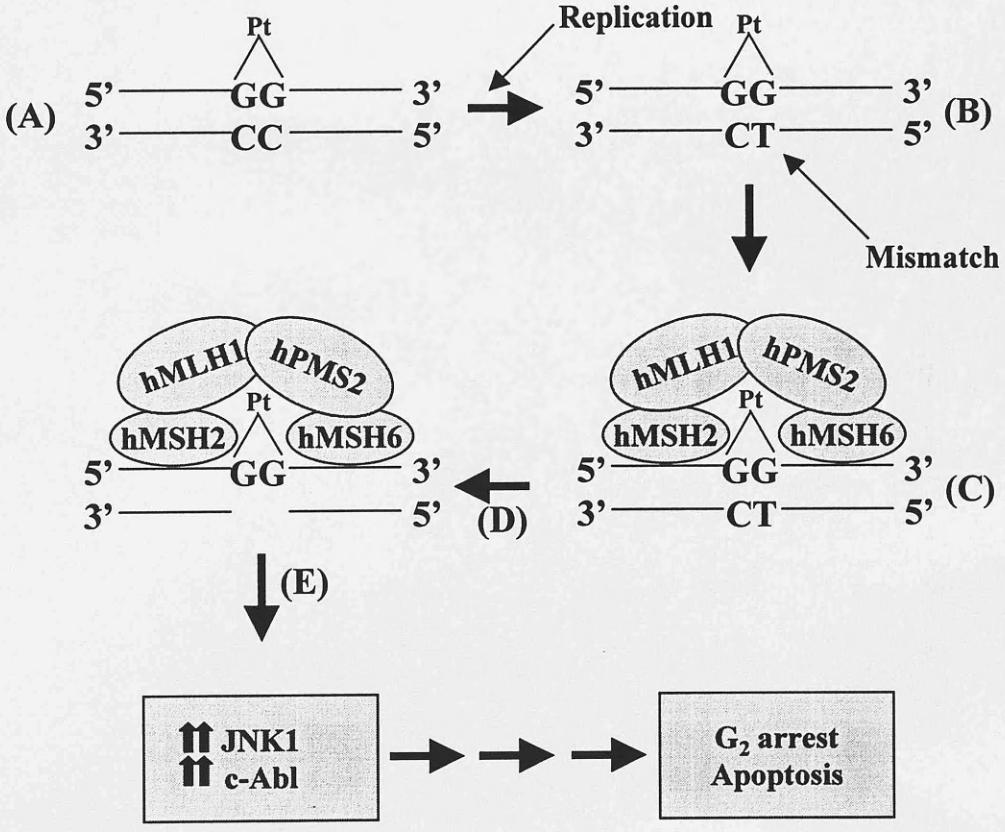
Recently, MMR has been implicated in cellular processes other than stabilising the genome after replication. These processes include: (a) the recognition of lesions other than conventional mispairs, such as cisplatin adducts, and (b) the participation in a cell-cycle checkpoint control system

which may trigger apoptosis (Hawn et al., 1995). These two alternative functions appear to be interconnected.

Several studies have documented that loss of MMR is an important mechanism of resistance to a variety of clinically important drugs, due to the fact that the MMR system can recognise and bind to various types of adducts in DNA as well as to mismatches. Rather than being a major effector of the removal of such adducts, the main role of the MMR seems to be as a detector of specific types of DNA damage in a way which is represented in Figure 9.

There exist a number of cell lines with known inactivating mutations in the MMR genes. The human colorectal adenocarcinoma cell line HCT-116, which is hMLH1 deficient due to a hemizygous mutation in hMLH1 resulting in a truncated non-functional protein, exhibits microsatellite instability and does not correct mismatches in cell-free extracts (Parsons et al., 1993; Boyer et al., 1995). Transfer of chromosome 3, on which the hMLH1 gene is located, into HCT-116 cells corrects the MMR defect, reverses the mutator phenotype and sensitises the cells to the methylating agent *N*-methyl-*N'*-nitro-*N*-nitroguanidine (MNNG), thus indicating that restoration of functional MMR abolishes methylation tolerance (Koi et al., 1994). Similarly, the human endometrial adenocarcinoma cell line HEC59, which is hMSH2 deficient due to different mutations in each of the two hMSH2 alleles (Boyer et al., 1995), is resistant to MNNG when compared to a subline into which chromosome 2, containing a wild-type copy of hMSH2 gene, has been transferred (Umar et al., 1997). Furthermore, the human endometrial adenocarcinoma cell line HEC-1-A,

Figure 9. Basic sequence of events that seem to be the basis for cytotoxicity of cisplatin. **(A)** Formation of the 1,2 d(GpG) intrastrand cross-link adduct; **(B)** misincorporation of a base (T) opposite the adduct at the next round of replication; **(C)** recognition of the adduct/mispair by the mismatch repair (MMR) system; **(D)** attempted futile repair; **(E)** generation of a signal that triggers apoptosis.

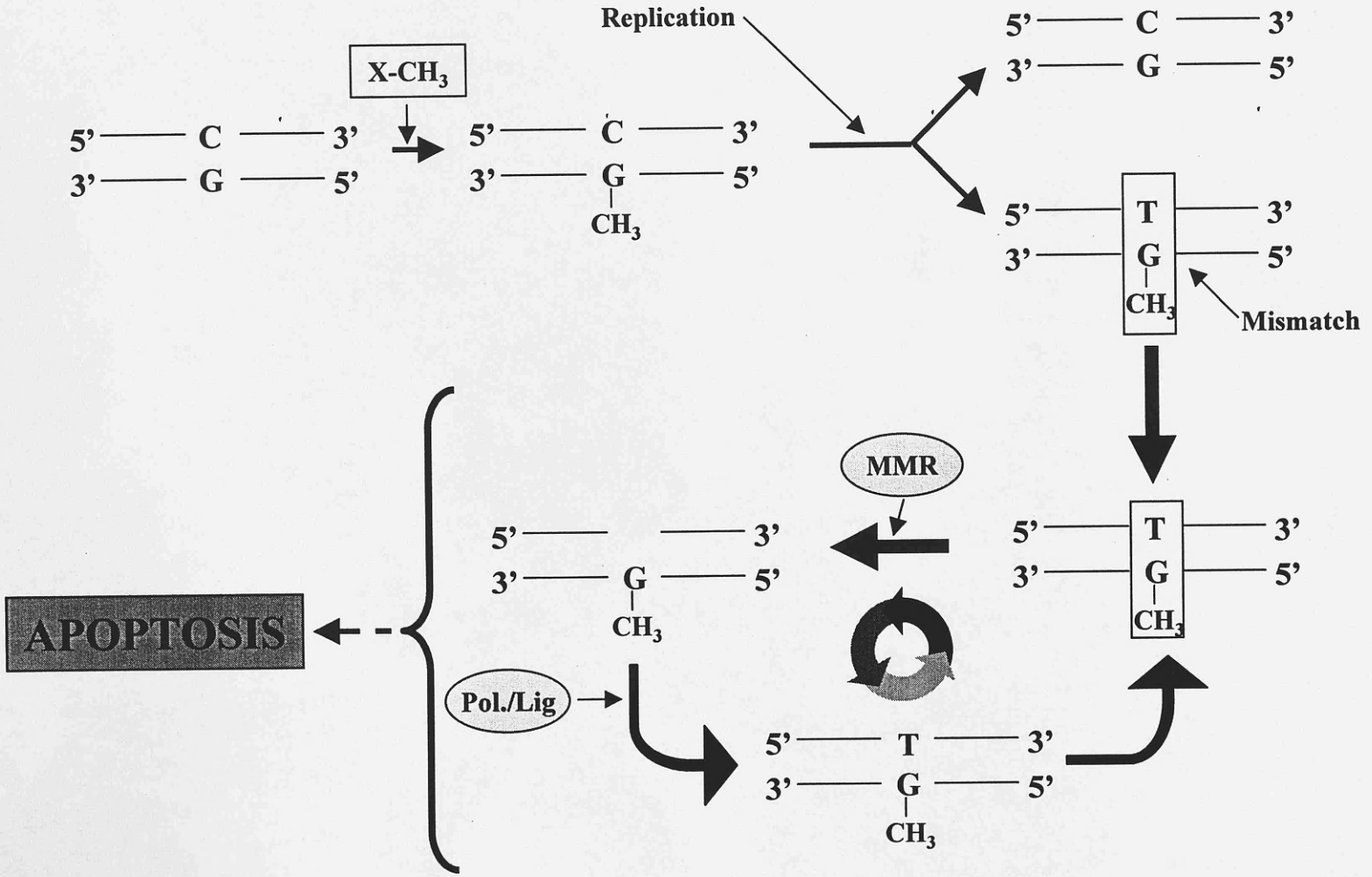

(A)
Pt
5'
GG
3'
3'
CC
5'
Replication
(B)
Pt
5'
GG
3'
3'
CT
5'
Mismatch
(C)
hMLH1
hPMS2
Pt
hMSH2
hMSH6
5'
GG
3'
3'
CT
5'
(D)
hMLH1
hPMS2
Pt
hMSH2
hMSH6
5'
GG
3'
3'
5'
(E)
JNK1
c-Abl
G2 arrest
Apoptosis

-Figure 9-

which carries mutations in hPMS2, exhibits microsatellite instability and resistance to MNNG when compared to the MMR-proficient KLE cells (Risinger et al., 1995).

Methylating agents such as *N*-methyl-*N*-nitrosourea (MNU), MNNG, procarbazine and temozolomide form a variety of adducts in DNA, among which O^6-methylguanine is the most toxic. Although the MMR system does not seem to be able to recognise the alkylated guanine directly (Moggs et al., 1997), it does recognise the O^6-methylguanine-thymine mispair that occurs after erroneous incorporation of a thymine rather than a cytosine opposite the O^6-methylguanine during the next cycle of DNA replication (Griffin et al., 1994). One hypothesis (Fig. 10) is that having recognised the mismatch, the MMR system incises the thymine-containing strand, excises the thymine and surrounding bases, creating a gap, and then fills in the gap via repair synthesis. However, because a thymine is again incorporated opposite the persisting O^6-methylguanine, the site is once again recognised by the MMR system, and a new round of attempted repair is triggered. This futile cycling is envisioned as increasing the risk of a double-strand break at the time of the next S phase that could then trigger apoptosis (Karran and Bignami, 1994). This model predicts that loss of MMR confers tolerance to methylating agents such as MNU and MNNG by virtue of the fact that the cell does not attempt repair and subsequent futile cycling. This hypothesis is supported by the evidence that selection of cells for resistance to MNU and MNNG frequently results in loss of MMR (Kat et al., 1993) and loss of guanine-thymine mismatch binding activity (Griffin et

Figure 10. Model for mismatch repair (MMR) and apoptosis activation connection. After the formation of the O^6-methylguanine adduct, an O^6-methylguanine-thymine mispair occurs after erroneous incorporation of a thymine rather than a cytosine opposite the O^6-methylguanine during the next cycle of DNA replication. The MMR pathway recognises the mismatch and MMR system incises the thymine-containing strand, excises the thymine and surrounding bases, creating a gap, and then fills in the gap via repair synthesis. However, because a thymine is again incorporated opposite the persisting O^6-methylguanine, the site is once again recognised by the MMR system, and a new round of attempted repair is triggered thus generating a futile cycling which could be able to increase the risk of a double-strand break at the time of the next S phase and, as consequence, to trigger apoptosis. Loss of MMR would be accompanied with loss of the futile cycling-mediated apoptosis and hence by increased cellular resistance.


Replication
X-CH3
5' C 3'
3' G 5'
5' C 3'
3' G 5'
CH3
5' C 3'
3' G 5'
5' T 3'
3' G 5'
CH3
Mismatch
MMR
5' 3'
3' G 5'
CH3
5' T 3'
3' G 5'
CH3
APOPTOSIS
Pol./Lig
5' T 3'
3' G 5'
CH3

-Figure 10-

al., 1994; Branch P. et al., 1993). Loss of MMR seems to be important as a mechanism of resistance to two drugs currently used in the clinic, both of which produce a large number of O^6-methylguanine adducts, namely temozolomide and procarbazine (Liu L et al., 1996; D'Atri et al., 1997; Friedman et al., 1997). There is an interesting interplay between MMR and MGMT, which is able to directly remove the methyl group from the O^6 position of guanine and which represents the major route for removal of these DNA-adducts. However, these alkylated guanines are only toxic to the cells if they are detected by the MMR system. For cells with normal MMR, high levels of MGMT prevent the drug-induced cytotoxic effect by removing the O^6-alkylguanine DNA adducts, and inactivation of this enzyme by a potent inhibitor (O^6-benzylguanine or fotemustine) sensitises cells to killing by temozolomide (Pegg et al., 1993). In MMR-deficient cell lines, inactivation of MGMT fails to sensitise cells to temozolomide, suggesting that even large numbers of DNA adducts are not cytotoxic in the absence of the MMR detector, although they may be very mutagenic. Thus, MMR mutations seem to override the MGMT mechanism of resistance (Liu L et al., 1996); if the cell cannot detect the presence of the adducts on DNA, then it matters less how many such adducts are present.

Several recent studies provide evidence that the MMR functional status is an important factor in determining cellular sensitivity to a variety of anticancer drugs other than MNU, MNNG and temozolomide. MMR deficiency due to the lack of hMSH2 or hMLH1 or hPMS2 expression was found to be associated

with resistance to the procarbazine (Friedman et al., 1997), DNA-minor groove monofunctional alkylating agents tallimustine, CC-1065 and carzelesin (Colella et al., 1999), 6-thioguanine (Aebi. et al., 1997; Hawn et al., 1995) and some of the platinum-based drugs. Even though cisplatin and its derivatives could not be considered classical alkylators, their capacity to covalently bind the DNA at N^7-purine nucleophilic sites has been extensively demonstrated.

It is known that DNA is the primary intracellular target of cisplatin and eukaryotic cells respond to the presence of cisplatin adducts in DNA by activating signal transduction pathways that result in cell cycle arrest and apoptosis (Eastman, 1990). The first indication that MMR might be a determinant of sensitivity to cisplatin was the observation that introduction of MutS or MutL mutations into E. coli (already hypersensitive to cisplatin due to the presence of a DNA adenine Methyltransferase (dam) mutation) caused them to become resistant (Fram et al., 1985). Subsequently it was demonstrated that in two unrelated cell systems, loss of either hMLH1 (HCT-116 cells) or hMSH2 (HEC59 cells) function resulted in low level cisplatin (Anthoney et al., 1996; Aebi et al., 1996; Drummond et al., 1996) and carboplatin resistance (Fink et al., 1996), and that some human tumour cell lines selected for resistance to cisplatin (2008/A, for example; Narcdi et al., 1995) exhibit a marked microsatellite instability and are defective in strand-specific MMR (Drummond et al., 1996). The fact that loss of MMR results in resistance to both cisplatin and carboplatin was not unexpected because although carboplatin contains a 1,1-cyclobutanedicarboxylato-leaving group and undergoes aquation

more slowly, the structures of aquated forms of cisplatin and carboplatin are the same as are the types of adducts.

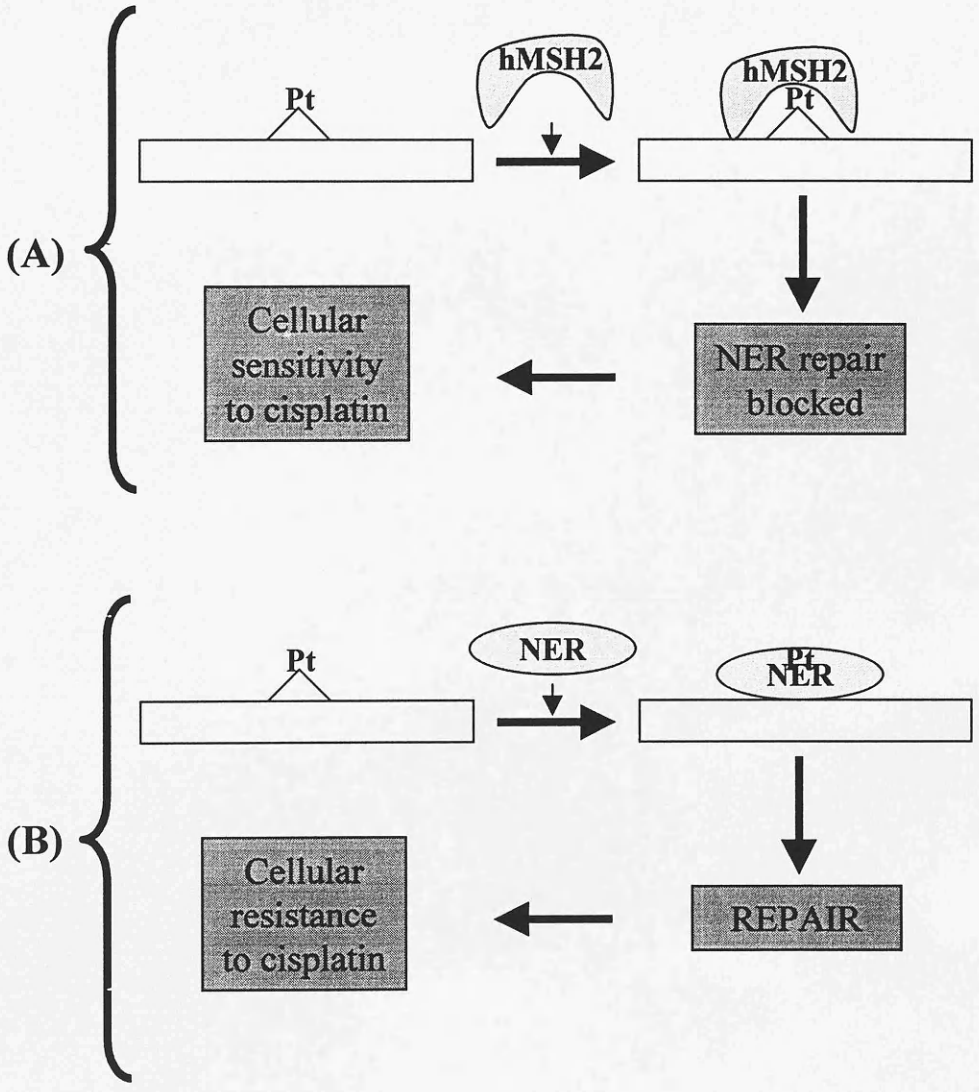
Pure hMSH2 has been reported to bind to platinated DNA in electromobility shift assays (Mello et al., 1996). Human MutSα, a heterodimer of hMSH2 and hMSH6, has also been shown to bind DNA containing adducts produced by cisplatin (Duckett et al., 1996) and has greatest affinity for lesions in which a thymine has been misincorporated opposite the 3' guanine (Yamada et al., 1997). It is interesting to note that adducts formed by other two cisplatin derivatives, oxaliplatin and transplatin, are not recognised by the MMR system (Fink et al., 1996; Duckett et al., 1996). These two drugs do not differ in their cytotoxicity to MMR-deficient and –proficient tumour cell lines (Fink et al., 1996), suggesting that the components of the MMR system responsible for the difference in sensitivity are quite specific in their ability to discriminate types of closely related DNA adducts.

It is not yet clear how the platinum adducts are recognised by the MMR proteins. It has been demonstrated biochemically that hMSH2 can bind 1,2 d(GpG) intrastrand cross-link adducts (which are the predominant DNA damages produced by platinum-based compounds), but it is also possible that the MMR system recognises monoadducts, monoadducts modified by the reaction with glutathione, or interstrand cross-link adducts as well. As hypothesised for monofunctional methylating agents and DNA-minor groove alkylators, also in this case it is possible that cisplatin adducts locally distort the DNA in a manner that mimics the presence of either a single-base mismatch or

an insertion/deletion mispair. The current paradigm is that, as for the methylating agents, the MMR system serves as a detector for cisplatin-damaged DNA: during the replication of DNA platinated guanine could not be able to form an hydrogen bond with its physiological partner thymine thus producing a mispair recognised by MMR proteins. Then, the MMR system removes unmodified thymine leaving platinated guanine which, after the reparative synthesis, is again unable to correctly bind cytosine. The site will again be recognised by MMR proteins and a new round of attempted repair will be performed starting a futile cycle which could trigger apoptosis (Fig. 10). In this context, resistance due to the loss of MMR system activity is thought to result from failure of the cell to recognise the cisplatin adducts and activate futile cycling and other signalling pathways that trigger apoptosis (Fink et al., 1998). An alternative hypothesis (Crul et al., 1997) for the involvement of the MMR proteins in cisplatin DNA adduct repair was postulated: by binding cisplatin-DNA adducts, hMSH2 may shield these adducts from repair (mainly performed by NER), thus allowing these adducts to persist. Loss of MMR would be accompanied with loss of the shielding by hMSH2 and hence by enhanced repair of such adducts, leading to increased resistance (Fig. 11). Both hypotheses for the involvement of the MMR proteins in the repair of cisplatin lesions are plausible. Since they do not necessarily exclude each other, the actual situation *in vivo* could be a combination of the two.

Data supporting the hypothesis of MMR system as a detector able to initiate activation of signalling pathways are emerging. It has recently been reported

Figure 11. An alternative model for the involvement of the mismatch repair (MMR) proteins in cisplatin DNA adduct repair. **(A)** By binding cisplatin-DNA adducts, hMSH2 may shield these adducts from repair (mainly performed by NER), thus allowing these adducts to persist. **(B)** Loss of MMR would be accompanied with loss of the shielding by hMSH2 and hence by enhanced repair of such adducts, leading to increased cellular resistance.


(A)
Pt
hMSH2
hMSH2
Pt
NER repair
blocked
Cellular
sensitivity
to cisplatin
(B)
Pt
NER
Pt
NER
REPAIR
Cellular
resistance
to cisplatin

-Figure 11-

that cisplatin activates c-jun NH_2-terminal kinase 1 by a p21-activated kinase protein 65 and a mitogen-activated protein kinase 4-independent mechanism more efficiently in MMR-proficient cells than in MMR-deficient cells. Moreover, cisplatin seems to be able to activate c-Abl kinase only in the MMR-proficient cells, since this response is completely absent in MMR-deficient cells (Nehmé et al., 1997). These findings reveal that activation of c-jun NH_2-terminal kinase 1 and c-Abl by cisplatin is in part dependent on the MMR functional status, and suggests that these kinases are part of the signal transduction pathway activated when MMR proteins recognise DNA adducts produced by alkylating and platinum-based agents.

Data obtained after exposure of MMR-proficient cells to both MNNG and 6-thioguanine, indicate the MMR system as being able to promote G_2 cell cycle arrest and cell death (Carethers et al., 1996; Hawn M.T. et al., 1995). In fact, exposure of MMR-deficient cells to these drugs did not induce a G_2 block but rather just a G_1 delay. The arrest at the G_2 cell cycle checkpoint may allow the cell to attempt repair of DNA mismatches and prevent the replication of mutated DNA. The signals generated by the detection of DNA lesions by the MMR system are unknown, although there is some evidence that they work via regulation of the $p34^{cdc2}$-cyclin B1 complex (King et al., 1994). Very recently it has been reported that hMLH1-deficient tumour cell lines also fails to engage G_2 cell cycle arrest after cisplatin damage (Brown et al., 1997). These data altogether indicate the possibility that the MMR system is linked to the G_2 checkpoint thus suggesting that the MMR system is involved not only in the

repair of true mismatches but also in processes that limit the replication of cells when DNA damage is detected. In addition, it has been reported that MMR can cooperate with p53. In fact, studies performed in human coloncarcinoma HCT-116 cell line evidenced that lack of hMLH1 enhances the role of p53 in protecting the cells from cisplatin-induced DNA damage (Vikhanskaya et al., 1999).

Overall such evidence indicated that loss of MMR can result in drug resistance both directly, by impairing the ability of the cells to detect DNA damage, and indirectly, by increasing the mutation rate at loci that mediate resistance to other classes of drugs. Although, by the direct route, loss of the MMR pathway results only in a relatively small degree of resistance to platinum-containing drugs, several lines of evidence suggest that this resistance is nevertheless of substantial biological and clinical significance. Specifically: (a) this low-level resistance to cisplatin has been reported to be sufficient to produce progressive enrichment for MMR-deficient tumour cells during treatment *in vitro* (Fink et al., 1997); (b) $MSH2^{+/+}$ embryonic stem cells (de Wind et al., 1995) grown as xenografts have been shown to be responsive to cisplatin treatment whereas isogenic $MSH2^{-/-}$ tumours were not, suggesting that the degree of cisplatin resistance conferred by loss of MMR is sufficient to produce a large difference in clinical responsiveness *in vivo* (Fink et al., 1997); (c) loss of MMR has been reported in tumour cell lines selected for resistance to both cisplatin (Aebi et al., 1996) and doxorubicin (Drummond et al., 1996); (d) the frequency of positive immunoblot analysis for hMLH1 protein in

ovarian carcinomas obtained after chemotherapy with a cisplatin or carboplatin-containing regimen was shown to be substantially lower than the frequency observed in tumours sampled before treatment (Brown et al., 1997). Although the samples were not paired, this loss of hMLH1 expression is consistent with the concept that treatment with platinum drugs can enrich for MMR-deficient cells.

The issue of when loss of MMR occurs during oncogenesis remains controversial even for hereditary nonpolyposis colon cancer, which represents the best-defined clinical situation (Tomlinson et al., 1996). However, once such cells are present in the tumor, their genomic instability may result in the accumulation of additional mutations that contribute to of tumour progression. Enrichment of these cells as a result of chemotherapy would be expected to accelerate this process. Indeed, microsatellite instability, a hallmark of the genomic instability due to loss of MMR (Loeb, 1994), has been reported to be present in up to 94% of the patients with therapy-related leukemia or myelodysplastic syndromes, consistent with drug-induced enrichment for genetically unstable cells (Ben-Yehuda et al., 1996).

1.3.3.4 Connection between MMR and Transcription-coupled NER

Since alterations in NER and MMR pathways determine opposite effects on the cellular response to cisplatin, it is plausible that the balance between the activities of these two systems can be an important factor in determining the sensitivity profile of tumour cells to DNA-damaging anticancer drugs.

Recently, it was found that in E. coli carrying mutations in MMR genes, rapid repair of the transcribed strand of active genes after UV damage was selectively abolished (Mellon and Champe, 1996). This implicates an association between MMR and NER, i.e components of DNA MMR system probably function in transcription coupled repair (TCR) as well. It was also shown that this connection exists in eukaryotes, since several MMR-deficient human tumour cell lines, characterised by mutations in the hMSH2, hPMS2 and hMLH1 genes, demonstrated the same deficiency in TCR as the E. coli mutants (Mellon et al., 1996). In addition, TCR was restored in a mutant human cell line whose MMR-deficiency had been corrected by chromosome transfer (Mellon et al., 1996). The following working model was proposed to explain these findings (Mellon and Champe, 1996): RNA polymerase II stalls at a lesion in the template strand, thereby altering the conformation of the DNA. Some features of this altered DNA of the surrounding region might resemble a mismatched heteroduplex to which the MMR proteins could bind. TRCF would then displace the stalled RNA polymerase II. The lesion is

stabilised at a distance by the MMR proteins and is available for recognition by the NER proteins.

The implications of a connection between MMR and TCR-NER for cisplatin therapy are not clear. The MutSα complex binds cisplatin 1,2-intrastrand cross-links, but it does not recognise the 1,3-intrastrand adducts (Duckett et al., 1996). However, as mentioned above, it is still not clear which of the cisplatin DNA adducts is the most toxic lesion.

1.3.3.5 Structure-specific damage-recognition proteins (DRPs).

The first step in DNA repair is the recognition of the damage. A wide variety of proteins are involved in this process. These proteins probably do not recognise specific adducts, but merely bind to sites where damage has caused more or less specific conformational changes in DNA (Bruhn et al., 1992; Donahue et al., 1990). Main goals in recent research were to identify and characterise DRPs, and to investigate a possible correlation between their relative levels of expression in tumour cells and the sensitivity of these cells to chemotherapy. For cisplatin, the DRPs of the NER system are of importance, but also other proteins that bind to platinated DNA have been identified (Tab. III). Many of these DRPs contain a 'high-mobility group' (HMG) domain. The HMG domain (also referred to as HMG-box) (Grosschedl et al., 1994) is a DNA-binding motif such as zinc finger, helix-turn-helix or leucine zipper (McA'Nulty and Lippard, 1996a). The HMG domain is found in a large

Table III. Structure specific damage recognition proteins for 1,2-intrastrand cisplatin-DNA adducts.

Protein	General features	Overexpression in cisplatin-resistant cells
Ixr1	Yeast protein, contains two HMG domains	?
HMG1	28 KDa, contains two HMG domains	No
HMG2	26.5 KDa, contains two HMG domains and is highly homologous to HMG1	No
SSRP1	81-100 KDa, contains one HMG domain	Yes?No?

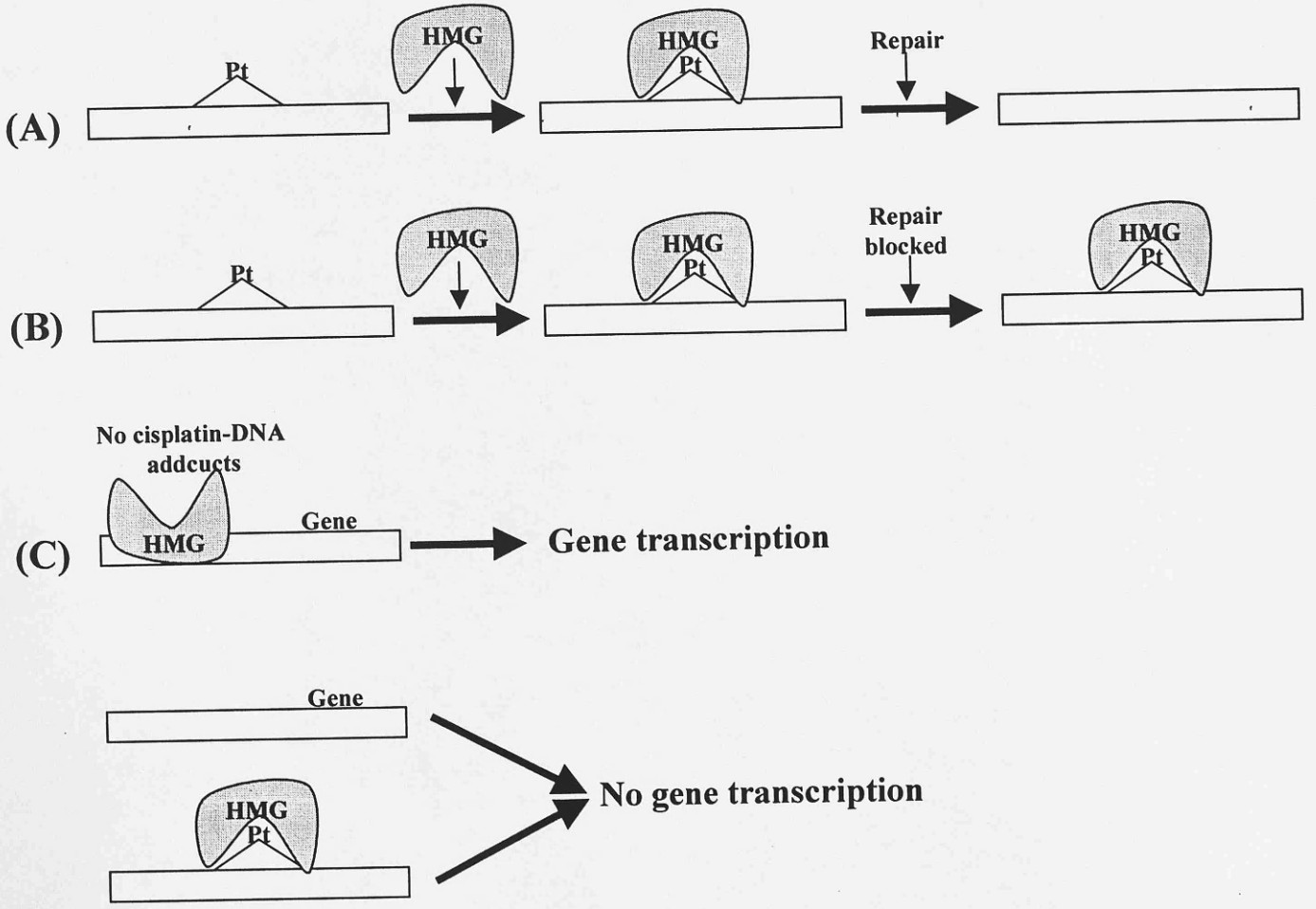
-Table III-

number of proteins (McA'Nulty and Lippard, 1996a). HMG-domain-containing proteins have been divided into two subfamilies based upon their ability to bind DNA in a sequence-specific versus structure specific manner (Grosschedl et al., 1994). The HMG-domain-containing proteins that recognise cisplatin adducts all belong to the second category. Most of the HMG-domain proteins tested to date bind DNA modified with cisplatin but not with transplatin (Zamble and Lippard, 1995). In addition, the binding of HMG-domain proteins is specific for the 1,2-intrastrand adducts (Zamble and Lippard, 1995). The 1,2-intrastrand adducts unwind the DNA duplex by 13° and cause a 34° kink in the direction of the major groove (Anin and Leng, 1990). Other adducts, not recognised by HMG-domain proteins, cause different degrees of bending and unwinding (Visse, 1994; Anin and Leng, 1990). Thus, it seems likely that the HMG-domain proteins recognising the 1,2-d(GpG) and 1,2-d(ApG) cisplatin–DNA intrastrand adducts are specific for their typical distortion of the DNA helix. It is highly unlikely that human cells encode a binding protein specific for cisplatin-damaged DNA. Thus, the HMG-domain proteins must have another, as yet unidentified, intrinsic function in the cell. It is speculated that they are involved in transcription (McA'Nulty and Lippard, 1996a). This could occur by direct activation of transcription of the regions accessible to transcription or by assisting factors involved in transcription to bind DNA and activate RNA polymerase II (McA'Nulty and Lippard, 1996a). For the interactions between HMG-domain proteins and cisplatin-damaged DNA, as well as for the effects of HMG-domain proteins binding to cisplatin adducts on the biological activity

of cisplatin, several hypotheses have been postulated (Fig. 12). Initially it was thought that when HMG-domain proteins bind to the cisplatin DNA adducts, they serve as recognition elements for repair, thus recruiting other proteins in the NER cascade to the site of damage (Fig. 12a). However, this hypothesis was proven not to be valid (Brown et al., 1993; McA'Nulty and Lippard, 1996b). The second model proposes that HMG-domain proteins bind tightly to cisplatin-DNA adducts and prevent access of the repair complex to the site of the damage shielding the cisplatin adducts from the NER proteins (Fig. 12b). The third possibility is that the cisplatin lesions titrate the HMG-domain proteins away from their natural binding sites, thereby decreasing the ability to perform their natural function in the cell (Fig. 12c).

In yeast, a protein that binds platinated DNA was identified and named Ixr1 (Brown et al., 1993; McA'Nulty and Lippard, 1996b). The protein contains two HMG domains and is able to bind 1,2-intrastrand cross-links specifically. Moreover, Ixr1 did not bind unmodified DNA or DNA modified with transplatin (Brown et al., 1993). Disruption of the IXR1 gene resulted in increased resistance to cisplatin (Brown et al., 1993; McA'Nulty and Lippard, 1996b) thus indicating that in mutant cells lacking Ixr1, the shield is absent, thereby allowing repair to occur. Notably, it was found that disruption of the IXR1 gene did not alter the sensitivity of yeast to other DNA-damaging agents such as UV irradiation, but that the effect was specific for cisplatin (Brown et al., 1993).

Figure 12. Possible mechanism for the interactions between cisplatin-DNA adducts and HMG-domain proteins. **(A)** HMG-domain proteins act as damage recognition elements and enhance repair; **(B)** HMG-domain proteins shield the cisplatin-DNA adducts from the repair proteins; **(C)** the cisplatin-DNA adducts titrate the HMG-domain proteins away from their natural binding sites, thereby decreasing the ability to perform their natural function in the cell.


(A)
Pt
HMG
HMG
Pt
Repair
(B)
Pt
HMG
HMG
Pt
Repair
blocked
HMG
Pt
(C)
No cisplatin-DNA
addcucts
HMG
Gene
Gene transcription
Gene
HMG
Pt
No gene transcription

-Figure 12-

Multiple studies identified two HMG-domain proteins in human cells, which were designated HMG1 and HMG2 (Szymkowski et al., 1992; Huang et al., 1994; Bissett et al., 1993; Clugston et al., 1992; McLaughlin et al., 1993; Billings et al., 1994; Hughes et al.; Marples et al., 1994; Pil and Lippard, 1992; Toney et al., 1989). The difference between HMG1 and HMG2 is in the size; HMG2 (26.5 KDa) is somewhat smaller than HMG1 (28 KDa) (Hughes et al., 1992). These two proteins are able to bind 1,2-d(GpG) and 1,2-d(ApG) intrastrand cross-links but do not bind 1,3-d(GpG) intrastrand adducts (Pil and Lippard, 1992). As mentioned above, the fact that these latter cisplatin DNA lesions are repaired more efficiently than 1,2-intrastrand adducts (Huang et al., 1994) is a further support to the theory that HMG-domain proteins shield the cisplatin adducts from the NER machinery. In unmodified DNA, higher affinity for single-stranded DNA over double-stranded DNA for HMG-domain proteins has been described (Isackson et al., 1979); this is consistent with a role for these proteins in transcription initiation since single-stranded DNA is required for transcription. However, in platinated DNA, similar levels of HMG1 and 2 binding to double-stranded and single-stranded DNA was observed (Hughes et al., 1992). Levels of HMG1 and 2 proteins were shown to increase in a dose dependent manner in parental HeLa cells after exposure to different levels of cisplatin (Hughes et al., 1992). No changes in the expression of these two proteins have been found in resistant cells (as compared to the sensitive counterpart) derived from ovarian, testicular and bladder tumours (Bissett et al., 1993; Chao et al., 1991a; McLaughlin et al., 1993).

To investigate the possibility that cellular sensitivity to platinum compounds is related to binding activity of the HMG1 and 2 proteins to DNA adducts, rather than to absolute levels of these proteins, these binding activities were studied in ovarian tumour cells derived from twelve different patients (Bissett et al., 1993). No correlation between binding activity and drug sensitivity was observed. However, another study reported increased levels of HMG-binding activity as a function of sensitivity of the investigated cells to cisplatin treatment (Billings et al., 1994); intrinsic differences in the cell lines used in the two studies may explain the observed discrepancy.

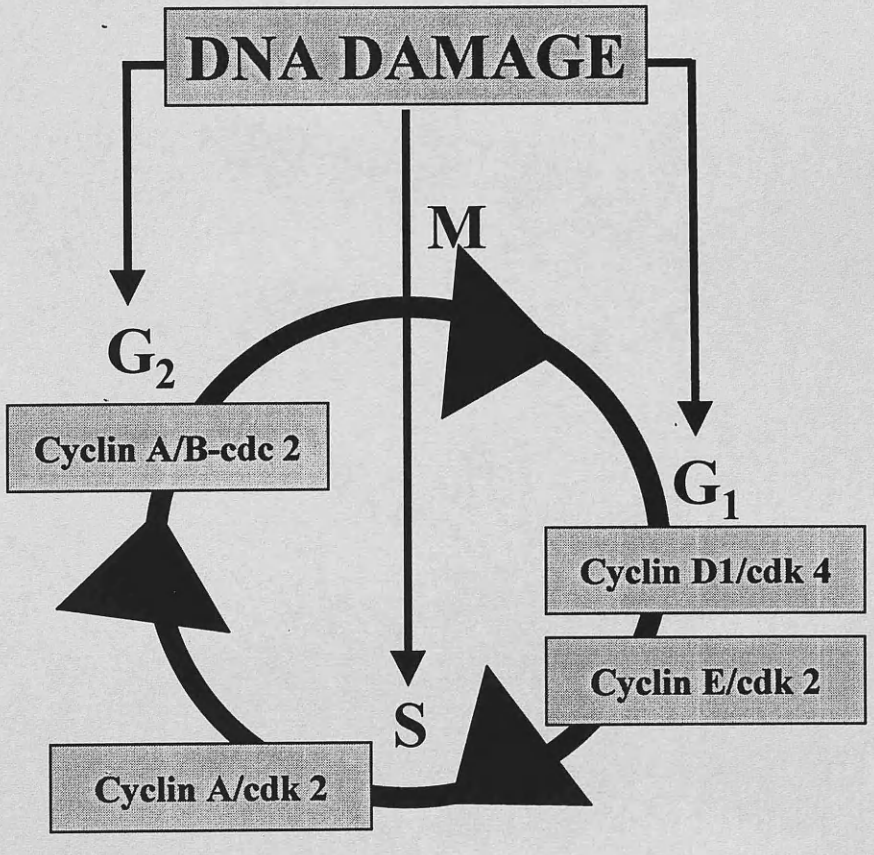
Another DNA damage-recognition protein has been identified in HeLa cells (Toney et al., 1989), and human testicular and bladder tumor cells (McLaughlin K. et al., 1993). After the isolation of its encoding human cDNA (Bruhn S.L. et al., 1992), the protein was named SSRP1 (structure-specific recognition protein 1). The SSRP1 protein contains one HMG domain. As with the other DRPs, the protein binds only 1,2-intrastrand cisplatin DNA adducts and not 1,3-intrastrand lesions. Moreover, the protein does not recognise adducts formed by transplatin or DNA damage caused by UV exposition. Higher levels of such a protein were found in cisplatin resistant testicular tumour cells with respect to their sensitive counterpart (McLaughlin et al., 1993). Conversely, in studies carried out on other tumor cell lines, no differences were observed (McLaughlin et al., 1993; Bruhn et al., 1992; Donahue et al., 1990). Moreover, the protein was not inducible after cisplatin exposure (Bruhn et al., 1992). As yet, the natural function of SSRP1 is still unclear.

Several other HMG-domain-containing proteins have been described (sex determining region Y SRY, human upstream binding factor hUBF, mithocondrial transcription factor II MTFII). However, involvement of such proteins in the recognition of cisplatin DNA damage and cisplatin toxicity still remains to be determined.

1.4 Cell cycle progression and apoptosis

The life cycle of an eukaryotic cell consists of four phases: mitosis (M) and DNA synthesis (S), and the two periods in between gap1 (G_1) and gap2 (G_2). When DNA is damaged, progression of the cell cycle to replication (S phase) could lead to mutations in the daughter DNA. To counteract this mutation threat, cells employ mechanisms to arrest the cell cycle temporarily when DNA is damaged, probably to allow repair to occur. Cell cycle arrest is governed by a series of control systems called 'checkpoints' (Enoch and Norbury, 1995). Activation of these checkpoints provides the cells with additional time to complete DNA repair (Fig. 13). However, if the damage appears to be irreparable, the cell can undergo apoptosis (Bedford. et al., 1988). In these processes, the p53 protein plays a key role. After DNA damage, the p53 protein is thought to bind DNA and to activate or regulate multiple genes involved in the control of cell cycle progression, in DNA repair pathways and in apoptosis. It has been suggested that replication as well as cell cycle arrest are necessary for induction of apoptosis (Anthoney et al., 1996; Sorenson and Eastman,

Figure 13. Mammalian DNA damage checkpoints. DNA damage induces the arrest of the cell cycle progression in G_1 and/or G_2 phases. Delayed progression through S phase has also been noted for some DNA damaging agents. Cell cycle arrest is mediated by a series of control systems commonly termed checkpoints (composed by cyclin and cyclin-dependent kinases) able to prevent the formation and/or activation of the complexes which can be thought of as the engine of the cell cycle.


DNA DAMAGE
M
G2
Cyclin A/B-cdc 2
G1
Cyclin D1/cdk 4
Cyclin E/cdk 2
S
Cyclin A/cdk 2

-Figure 13-

1988). However, at extremely high levels of DNA damage , apoptosis has been described without prior cell-cycle arrest or replication (Sorenson and Eastman, 1988; Boulikas, 1996).

Replicative bypass is defined as the ability of the replicative complex of a cell to synthesise DNA past the site of DNA damage. Bypassing of cisplatin-DNA adducts during the S-phase of the cell cycle would allow a cell to progress into G_2 in which it could arrest and repair the damaged sites before proceeding into mitosis (Brown, 1995). Enhanced replicative bypass has been observed in four different cisplatin resistant human ovarian cancer lines as well as in rodent cell lines as compared to cisplatin-sensitive cell lines (Gibbons et al., 1991; Mamenta et al., 1994). Research into the mechanism of replicative bypass demonstrated that only DNA polymerase β is able to bypass the cisplatin DNA 1,2-d(GpG) intrastrand adduct. Moreover, DNA polymerase β was able to elongate the arrested replication products of DNA polymerases α, δ and ε (Hoffmann et al., 1995). The main difference between DNA polymerase β and other polymerases is believed to be the ability of the former to frequently dissociate and re-associate with the DNA strand, and to catalyse extension from the position upstream of the cisplatin DNA adduct, probably by reinitiating replication at the site opposite to the adduct (Hoffmann et al., 1995). Induction of DNA polymerase β has been observed in CHO cells after exposure to various DNA-damaging agents but not after treatment with cisplatin (Fornace et al., 1989). In addition, a 3 to 5-fold increased levels of DNA polymerase β mRNA were found in the cisplatin resistant human ovarian cancer cell line

A2780cp8 with respect to its cisplatin sensitive counterpart A2780 (Scanlon et al., 1989a,b; Scanlon and Kashani-Sabet, 1989; Valentinis et al., 1993).

In recent years several studies were performed on different model systems including yeast, frog, mouse and human cells to better understand the mechanisms governing DNA damage-induced cell cycle arrest. In mammalian cells, cells cycle progression is halted in G_1 and/or G_2 phases following DNA damage and, for certain DNA damaging agents, a prominent S phase delay has also been reported (Brown, 1995). As mentioned above, delay at these points in the cell cycle appears to be governed, in large part, by a series of checkpoints (Hartwell, 1992; Murray, 1992; Hartwell and Kastan 1994). Activation of these DNA damage checkpoints has been proposed to extend the period of time for DNA repair to occur before entry into S phase or mitosis. This in turn would suppress the replication of damaged DNA templates and the likelihood of their correct segregation into daughter cells. Cell cycle arrest can thus contribute to the fidelity with which genetic information is passed from one generation to the next, and the loss of these control systems can often have dramatic consequences on genome stability and cell survival following genotoxic stress (Livingstone et al., 1992; Yin et al., 1992; Hartwell., 1992; Hartwell and Kastan, 1994).

Cells exposed to cisplatin have been described to arrest in G_1 as well as G_2 phases (Sorenson et al., 1990; Evans and Dive, 1993; Fan et al., 1994). G_1 arrest is regulated by the product of the p53 gene (Fan et al., 1994). p53 could

also be involved in the regulation of G_2 cell cycle block although wild-type p53 function does not seem to be required for G_2 arrest following DNA damage (Kastan et al., 1991; Kuerbitz et al., 1992; O'Connor et al., 1993a). After cisplatin treatment, arrest in G_2 appears to be more obvious than of G_1 (Evans and Dive, 1993). Multiple studies have investigated, mostly using flow cytometric analysis, the cell cycle arrest caused by cisplatin and the underlying mechanisms. The duration of the G_2 block was proportional to the concentration of cisplatin to which the cells were exposed (Ormerod et al., 1994). In addition, a slower migration through S-phase was observed (Ormerod et al., 1994). These studies demonstrated that in order to reach G_2, where arrest for repair is possible, replication of the DNA-damaged template is a prerequisite (Ormerod et al., 1994), thus stressing the importance of replicative bypass.

In yeast and humans, cisplatin was found to induce a G_2 arrest, and this arrest was mediated by the product of a gene named wee1. The pathway leading to cell cycle arrest was kinase dependent and it was demonstrated that cells more resistant to cisplatin showed more pronounced arrest in G_2, whereas cells hypersensitive to this agent failed to show such an arrest (Thiebaut et al., 1994). Hence, the ability of the cell to arrest the cell cycle in G_2 might be a major determinant of resistance to cisplatin.

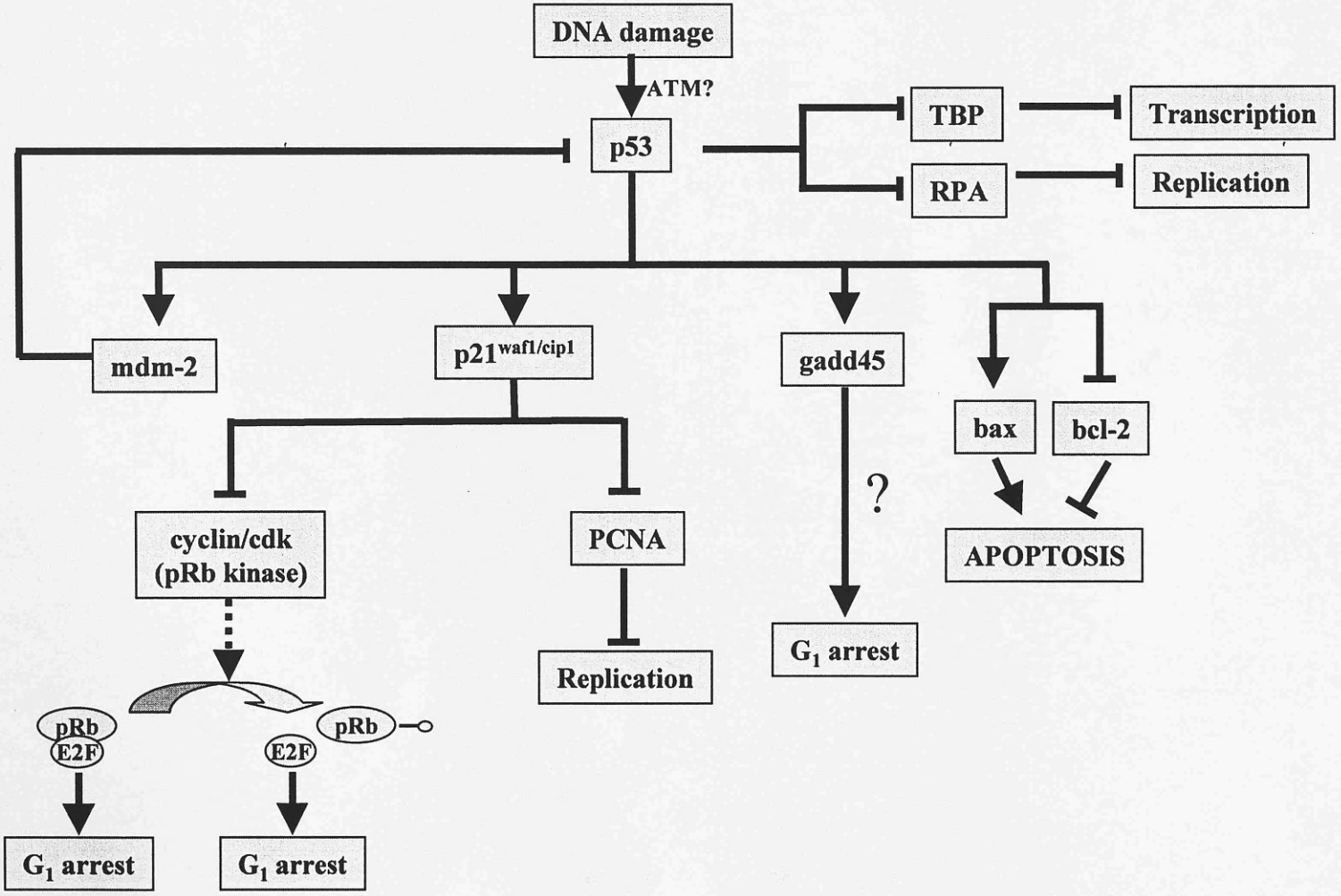
1.4.1 p53

The tumour suppressor gene p53 encodes a nuclear phosphoprotein that is formed by 393 amino acids and can be divided into at least 3 domains: an amino-terminal acidic transactivating domain, a central evolutionarily conserved DNA binding domain and a complex carboxy-terminal domain that houses potential nuclear localisation sequences, a homotetramerisation domain and a putative DNA damage recognition domain. p53 is the most commonly mutated gene found in human tumours. The majority of mutations are missense mutations that are clustered into the central DNA binding domain (Friend 1994; Greenblatt et al., 1994). Since the p53 protein can function as a sequence-specific DNA-binding protein that regulates gene expression, either positively or negatively (Volgelstein and Kinzler, 1992), these mutations block p53 DNA binding and transactivating activity and have frequently been termed "loss-of-function" mutations (Zambetti and Levine, 1993). p53 mutations are commonly associated with cellular accumulation of the p53 protein and evidence exists of a possible "gain-of-function" for certain p53 mutations (Zambetti and Levine, 1993). p53 also has the capacity to repress transcription of genes containing TATA elements (Mack et al., 1993). When DNA is damaged, the levels of p53 are increased, probably by stabilisation of the protein rather than by increased gene expression (Fritsche et al., 1993). High levels of p53 induce a G_1 arrest by transcriptional activation of genes involved in the G_1 cell cycle checkpoint. Loss of p53 may thus contribute to the genomic

instability common in tumour cells, both by allowing tumour cells to replicate damaged DNA and by promoting the survival of cells with genomic alterations accumulating with time (Miyashita and Reed, 1995). Many different DNA-damaging agents, such as ionising radiation, UV radiation and chemotherapeutic drugs, including cisplatin, can induce p53 (Zhan et al., 1993). In turn, p53 transactivates a number of different genes (Fig. 14) including GADD 45, encoding a protein involved in DNA repair (Hollander et al., 1993); $p21^{WAF1/CIP1}$, encoding a cyclin-dependent kinase inhibitor involved in G_1 and G_2 cell cycle arrest (Butz et al., 1995); MDM2, encoding an inhibitor of p53 transcriptional activation and a promoter of p53 deregulation (Fajac et al., 1996; Kondo et al., 1995); and BAX (Miyashita and Reed, 1995; Eliopoulos et al., 1995), encoding a promoter of apoptosis. Moreover, p53 has also been involved in the negative regulation of some proteins, such as the apoptosis inhibitor bcl-2 (Haldar et al. 1994).

It has been shown that DNA strand breaks are necessary and sufficient for DNA damaging agents to activate p53 (Nelson and Kastan,1994). This might explain why DNA base damaging agents like nitrogen mustard and cisplatin induce p53 relatively slowly compared to gamma-irradiation or the topoisomerase II inhibitor, etoposide (Fan et al., 1994). Previous studies performed showed that p53 dependent G_1 arrest could be induced by nuclear injection of linearised plasmid DNA. This was the case whether the DNA was cut by enzymes that produced blunt ends, 5'- or 3'overhangs. p53 dependent G_1 arrest was also observed after injection of circular DNA plasmid containing a

Figure 14. Scheme for the different scenarios that follow DNA damage-induced p53 activation. Upon DNA damage, p53 becomes active and can trigger several cellular events via two distinct and parallel pathways: transcription-independent and –dependent pathways. Via transcription-independent pathway, p53 may lead to DNA-repair or apoptosis. The transcription-dependent process is more elaborated and involves activation, probably in a simultaneous fashion, of several target genes (MDM2, $p21^{WAF1/CIP1}$, Bax, GADD 45 etc) that may lead to different outcomes such as degradation of p53, apoptosis, cell cycle arrest and DNA repair.


DNA damage
ATM?
p53
TBP
Transcription
RPA
Replication
mdm-2
p21waf1/cip1
gadd45
bax
bcl-2
?
cyclin/cdk
(pRb kinase)
PCNA
APOPTOSIS
Replication
G1 arrest
pRb
E2F
pRb
E2F
G1 arrest
G1 arrest

-Figure 14-

large gap (2.9 kb gap) or single-stranded circular DNA whereas with supercoiled, nicked or small gap (25bp gap)-containing circular DNA plasmid it was not. Again, the length of the linear DNA was also important for p53 activation since a 49 bp oligonucleotide was effective at inducing G_1 block but a 27 bp fragment or single-stranded oligonucleotides up to 49 nucleotides in length did not induce G_1 arrest (Nelson and Kastan 1994; Huang LC et al., 1996).

Recent studies have suggested that p53 might itself act as the direct DNA damage sensor (Jayaraman and Prives, 1995; Lee et al., 1995). p53 seems to have the ability to recognise and stably associate, *in vitro*, with short single-stranded DNA regions (<40 nucleotides) and insertion/deletion mismatch lesions. However, the fact that short single-stranded oligonucleotides (17-49 bases) did not induce G_1 arrest when microinjected into the nucleus of a wild-type p53 line (Huang LC et al., 1996) suggests that other components, such as ATM gene product (Savitsky et al.,1995; Kastan et al.,1992), might have to assemble with p53 on to the damaged DNA template to activate G_1 arrest.

1.4.2 p53 and G_1 delay

Arrest of cell cycle progression in G_1 phase following DNA damage requires the function of the p53 tumour suppressor protein (Kastan et al., 1991; Kuerbitz et al.,1992). While cells with an intact p53 pathway exhibit γ-ray induced G_1 arrest, cells with p53 gene mutations, which occur commonly in

human cancer (Hollstein, 1991; Levine et al., 1991), or cells expressing viral oncogenes, whose products bind to and inactivate p53 function, lack G_1 arrest response to DNA damage (Kastan et al., 1991, Kuerbitz et al.,1992; Kessis et al., 1993; O'Connor et al.,1993b; Zambetti and Levine, 1993). As mentioned above, the mechanism by which p53 induces cell cycle arrest can best be understood from the transcriptional activation function of the p53 tumour suppressor (Pietenpol, 1994).

Among the several genes involved in p53-mediated G_1 delay, one gene product in particular has been focused upon as the means by which p53 induces such an arrest of the cell cycle: $p21^{WAF1/CIP1}$. This protein is a potent inhibitor of the activity of the cyclin dependent kinases (CDKs) encoded by the WAF1/CIP1 gene, the promoter of which contains p53 binding elements. The discovery that p21 was a potent inhibitor of the CDKs provided a potential mechanism by which p53 could inhibit the cell cycle engine in G_1 phase. After DNA damage, induction of p53 leads to transactivation of the $p21^{WAF1/CIP1}$ gene thus inducing an intracellular accumulation of this protein. By binding to cyclin/CDK complexes cyclin D/CDK4 and cyclin A/CDK2, p21 inhibits their kinase activity thus inducing growth arrest at G_1 phase (El-Deiry et al., 1993) (Fig. 14). In support of this proposition, it was found that cyclin E/CDK2 complexes were inactivated in a p53-dependent manner following DNA damage and that binding of p21 to this kinase correlated with its inactivation (El-Deiry et al., 1994; Dulic et al., 1994). p21 also binds to PCNA, proliferating cell nuclear antigen involved in DNA replication. This finding

suggests that p21 might coordinately regulate CDK and PCNA activity (Fig. 14). Several studies have been performed to understand the functional significance of such p21-PCNA binding. Data obtained thus far indicates that the primary purpose of such an interaction may not be to inhibit DNA replication per se but it is possible that p21 regulates some aspect of PCNA function in NER. Although in certain studies performed *in vitro* the role of p21 in determining NER activity was not clear (Flores-Rozas et al., 1994; Waga et al., 1994, Lin et al., 1994), recent investigations in human cells lacking p21 gene have revealed that such cells have reduced DNA repair activity and are more sensitive to agents that induce DNA damage repaired through the NER pathway (MacDonald et al., 1996), thus indicating that p21 could possess a dual role in both the G_1 checkpoint and DNA repair.

Besides p21, additional components are required in the p53-mediated G_1 arrest. The GADD45 gene product, the expression of which is induced by p53, has been shown to block re-entry of cells into the S phase suggesting that this protein might contribute to G_1 checkpoint regulation probably by stimulating the DNA resynthesis step of the NER pathway (Smith et al.,1996).

G_1 delay following DNA damage allows an extended period of time for DNA repair to occur before entry into the S phase. However, once DNA repair has been completed the cell, if required, must recover from this stasis and re-enter the cycle. To do so the cell must coordinate several events including inactivation of p53 and p21, and regeneration of active cyclin/CDK complexes. The transcriptional inactivation of p53 could take place through MDM2, a p53-

induced gene product that binds to the amino-terminal transactivating domain of p53 and blocks p53 transcriptional activity (Wu et al., 1993; Chen et al., 1994). Once p53 has been inactivated, the longevity of G_1 delay is then limited by the stability of the newly synthesised p53-regulated gene products that induce G_1 block.

1.4.3 The G2 delay

Several recent studies demonstrated that cells exposed to a variety of DNA damaging agents such as γ-irradiation, alkylating agents and cisplatin showed a marked accumulation in the G_2-M phase of the cell cycle. However, several lines of evidence suggest that the mechanisms by which the same agent induces a G_2 block can differ depending on the origin and the genetic and biochemical characteristics of the cellular model (O'Connor and Kohn, 1992; Hartwell and Kastan, 1994; Weinert and Harwell, 1988; O'Connor and Fan, 1996).

A current view of the G_2 checkpoint controlling the G_2 to M transition in drug-treated cells suggests a system of mutually interacting kinases and phosphatases (O'Connor and Fan, 1996; Kohn et al., 1994; Hoffman et al., 1993) which act at the level of cyclin B1/cdc2 complexes to maintain DNA damaged cells in G_2 phase.

In some of the earliest biochemical studies on the mammalian G_2 checkpoint it has been observed that DNA damage-induced G_2 arrest was associated with suppression of $p34^{cdc2}$ kinase activity. It was proposed that DNA damage

suppressed the removal of inhibitory phosphorylations from the threonine-14 and tyrosine-15 positions of cdc2, probably through suppression of the cdc2-activating phosphatase cdc25 and/or by up-regulation of the cdc2-inhibitory kinases Wee1 or Mik1.

Cdc2 and cdc25 have been suggested to interact in an autocatalytic-feedback loop to bring about rapid activation of the cdc2 kinase (Hoffmann et al., 1993). In this positive-feedback loop, cdc25 binds to a small fraction of the excess pool of cdc2/cyclin B1 complexes. As a result of cdc2 dephosphorylation the now active cdc2 would focus its initial kinase activity towards cdc25 itself. Hyperphosphorylation of cdc25 has been correlated with up-regulation of cdc25 activity (Hoffmann et al., 1993; O'Connor et al., 1994) which could in turn allow this relatively low abundance cdc25 protein the extra activity it might need to rapidly dephosphorylate the remaining hyperphophosrylated-cdc2/cyclin B1 complexes in G_2 cells (O'Connor et al., 1994). Given the ability of cdc2 to phosphorylate cdc25 one might expect that if cdc25 had productive access to cdc2 it would become hyperphosphorylated in G_2 arrested cells. Such a state could be achieved even if cdc2 was only transiently activated and then immediately down-regulated by Wee1/Mik1 kinases. Since cdc25 does not become hyperphosphorylated in G_2 arrested cells, cdc2-cdc25 interaction either does not occur or there must be an up-regulation of a cdc25-inhibitory phosphatase that rapidly converts cdc25 back into its hypophosphorylated state. Consistent with the possibility that the G_2 checkpoint prevents cdc25-cdc2 interactions it was found that the interaction between cdc2 and cdc25 did not

occur in DNA damaged G_2 arrested cells (O'Connor et al., 1994).

An additional mechanism contributing to G_2 block is the downregulation of cyclin B1. Investigations performed on the HeLa cell line showed that cyclin B1 mRNA and protein levels accumulate from very low levels at the G_1/S phase border to peak levels in G_2/M and most of this accumulation occurs as cells enter G_2/M phase (Pines and Hunter, 1989). It has been demonstrated that γ-irradiation of S phase synchronised HeLa cells suppresses cyclin B1 mRNA and protein accumulation probably by suppression of mRNA stability and promoter activity (Muschel et al.,1991; Muschel et al.,1993; Maity A. et al., 1995). Reduced levels of cyclin B1 would limit the level of cyclin B1/cdc2 complexes in G_2 arrested cells perhaps below the threshold level required for the mitotic entry. However, the evidence that forced expression of cyclin B1 in HeLa cells did not eliminate G_2 delay, indicates the possibility that an additional regulatory mechanisms could contribute to G_2 checkpoint control in this cell line probably by imposing cdc2-inhibitory phosphorylations in the DNA damaged cells (Lock and Keeling, 1993; Metting and Little, 1995)

These data altogether indicate that the G_2-M checkpoint control could be regulated by two independent mechanisms that might cooperate at the level of cyclin B1/cdc2 complexes (cdc2 inhibitory phosphorylation and suppression of cyclin B1 levels) although recent studies suggested a possible involvement of p53 in the G_2-M checkpoint control. The importance of p53 in controlling the G_2 checkpoint was evidenced by studies in which it was found that the cytotoxic activity of DNA damaging agents can be enhanced by agents that

disrupt G_2 checkpoint control preferentially in cells defective for p53 (Lau and Pardee, 1982; Fan et al., 1995; Powel et al., 1995; Russell, et al., 1995). Using inducible systems for wild-type p53 overexpression it was found that p53 can induce G_2 delay (Stewart et al., 1995; Agarwal et al., 1995). Thus, although wild-type p53 is not required for G_2 arrest following DNA damage, it might contribute an extra layer of protection to G_2 checkpoint control (Kastan et al., 1991; Kuerbitz et al., 1992; O'Connor et al., 1993b). In accord with this possibility, a number of workers have found that chemical inhibitors of G_2 checkpoint function are less effective at overriding G_2 block in cells with intact p53 function (Fan et al., 1995; Powel et al., 1995; Russell, et al., 1995).

These data, taken together, indicated a possible role for wild-type p53 in G_2 checkpoint but the mechanistic basis is presently unknown. It is likely that the p53-induced G_2 delay could be mediated by $p21^{WAF1/CIP1}$ which is able to inhibit cyclin b1/cdc2 kinase activity, albeit poorly (Harper et al., 1993, 1995). In addition to this possibility, p53, which also possesses transcriptional repression activity, when overexpressed, could down regulate components required for the G_2/M transition, such as cyclin B1.

The length of G_2 delay following DNA damage has been correlated with cell survival (Weinert and Hartwell, 1988; Hartwell and Weinert, 1989, McKenna et al., 1991). In these situations, cells that failed to arrest or cells that only briefly delayed progression were found to be more sensitive to DNA damage. Such findings complied with the paradigm that G_2 delay ensures for completion of DNA repair before cell division and that cells that enter in mitosis

prematurely are more likely to suffer the deleterious consequences of DNA damage (Hartwell and Kastan, 1994). However, several studies have indicated that prolonged G_2 arrest does not always translate into a survival advantage.

As mentioned above, G_2 cell cycle arrest and cell death seem to be promoted by the MMR system (Carethers et al., 1996; Hawn et al., 1995). It is likely that the arrest at the G_2 cell cycle checkpoint may permit the cell to attempt repair of DNA mismatches and prevent the replication of mutated DNA. To support this hypothesis, it has been demonstrated that hMLH1-deficient tumour cell lines were not able to engage G_2 delay after cisplatin damage (Brown et al., 1997), thus indicating a possible tight link between G_2 checkpoint control and the molecular pathways responsible for DNA damage detection and repair.

1.4.4 Apoptosis

After exposure to DNA damaging agents, cell death can occur by necrosis or apoptosis, which are morphologically and biochemically distinct processes. Whereas necrosis results from a loss of osmoregulation and is characterised by cell swelling and random DNA degradation by lysosomal enzymes at late stages, apoptosis is a programmed form of cell death, characterised by surface blebbing, cytoplasmic contraction, nuclear condensation, packing of cellular components within membranes prior to their budding from the cell as apoptotic bodies and chromatin cleavage by endogenous nucleases at an early stage (Eastman, 1990; Fajac et al., 1996; Wyllie, 1993). The chromatin is cleaved

into oligonucleosomal-length fragments, which can be visualised by gel electrophoresis, since these fragments produce a very characteristic 'ladder' on the gel (Fajac et al., 1996).

Although some cell types respond to wild-type p53 induction, consequent to DNA damage activation, by arresting in G_1 phase other cell types respond by undergoing apoptosis (Michalovitz et al., 1990; Martinez et al., 1991; Yonish-Rouach. et al., 1991; Lowe et al., 1993a,b; Clarke et al., 1993, Fan S. et al., 1994; 1995). The mechanistic basis for these cell type differences remains to be determined, however, some insights come from studies in cells overexpressing c-myc or E2F-1 (Wagner et al., 1994; Wu and Levine 1994; Qin et al., 1994). These transcription factors, along with the transcription factor b-myb (Lin et al., 1994), can drive cells into S phase in spite of active p53. Although apoptosis in S phase commonly results from this by-pass, S phase entry may not be essential, since p53 can induce apoptosis in c-myc deregulated cells arrested in G_1 by isoleucine withdrawal (Wagner et al., 1994).

Whether "priming" a cell for S phase, by activating genes required for S phase progression, is a sufficient signal for apoptosis remains to be investigated. Cancer cell types that do not normally undergo apoptosis following wild-type p53-overexpression may harbour deregulated E2F- or c-myc, or alternatively may combat apoptosis by alternative mechanism such as overexpression of bcl-2 (Walton et al., 1993) or suppression of bax induction (Zhan et al., 1994). Interestingly, p53-mediated apoptosis can be suppressed by growth factors which in turn produce a more stable G_1 arrest (Canman et al.,

1995). These results suggest that growth factor signals impinge upon the decision making process which determines G_1 arrest and/or apoptosis. the outcome of which is to sway judgement towards G_1 arrest and survival.

The bax gene, which encodes a product that counters bcl-2's ability to protect cells against apoptosis (Oltvai et al., 1993) is transcriptionally activated by wild-type p53 (Selvakumaran et al., 1994; Miyashita. et al., 1994; Miyashita and Reed, 1995). Interestingly, bax is only up-regulated in those wild-type p53 cell lines that commit to apoptosis following p53 activation (Zhan et al., 1994). Moreover, bcl-2 mRNA levels decline following p53 activation (Selvakumaran et al., 1994; Zhan et al., 1994). These findings suggest that p53-dependent apoptosis might arise as the balance between bax and bcl-2 alters. Enriching this model are recent results that indicate that bcl-X_L mRNA levels are also induced by wild-type p53 in cells that commit to p53-dependent apoptosis (Zhan et al., 1996). Like bcl-2, bcl-X_L protects against apoptosis (Boise et al., 1993). These results suggest that bax-induced apoptosis can be modulated independently of bcl-2 and that bcl-X_L might raise the threshold for bax-induced apoptosis. Despite these findings it is unclear how the apoptotic machinery becomes activated by bax. Also, studies into requirement of p53-dependent transactivation for apoptosis have yielded conflicting results with some studies suggesting that the transactivating activity of wild-type p53 may not be required for apoptosis in all cell types (Caelles et al., 1994).

p53 disruption not only abrogates G_1 arrest following DNA damage but it can also suppress apoptosis in susceptible cell types. Key experiments

performed on murine embryonic fibroblasts transformed with adenovirus E1A revealed that such cells were sensitised to apoptosis induced by ionising radiation and some chemotherapeutic agents and importantly, wild-type p53 function was required for this sensitisation (Lowe et al., 1993a). Experiments performed on different cell lines demonstrated that those with a functionally intact p53 pathway were, on average, more sensitive to ionising radiation, etoposide, nitrogen mustard and cisplatin than cell lines with mutant p53 (O'Connnor et al., 1993; Fan et al., 1994). The decreased sensitivity of the mutant p53 cell lines correlated with an evasion of p53-mediated apoptosis, illustrating that the mutant p53 lines had a survival advantage. Results obtained in different cellular models have tended to promote the single impression that p53 confers decreased sensitivity to DNA damaging agents. However, the cellular context and the type of DNA damaging agent now appear to be important in determining the outcome of p53 activation. In fact, in contrast to what is described above, most workers have failed to observe any impact of p53 disruption on the cellular sensitivity to DNA damaging agents in experiments performed on murine embryonic fibroblasts, colon carcinoma cells, breast cancer cells, and head and neck cancer cell lines (Brachman et al.,1993; Slichenmyer et al., 1993; Fan. et al., 1995; Powell et al., 1995). Moreover, a recent study carried out on human ovarian cancer cells suggested that the loss of functional p53 can increase cisplatin cytotoxicity in these cells, with loss of G_1/S checkpoint control and decreased cisplatin-DNA adduct repair (Pestell et al., 2000).

From the clinical point of view, of particular interest is the ability of cisplatin-induced DNA damage to trigger apoptosis. After exposure to cisplatin, DNA damage is believed to be a major trigger for apoptosis (Fan et al., 1995) and thus the rate of repair of cisplatin DNA adducts in cells treated with this drug may be related to the rate of apoptosis. Cisplatin has demonstrated induction of apoptosis in multiple tissue types (Havrilesky et al., 1995; Otto et al., 1996), In murine mammary and ovarian cells, it was demonstrated that the rate of apoptosis increased with increasing doses of cisplatin treatment (Marples et al., 1994). Furthermore, a study on mouse leukemia L1210 cells demonstrated a dual response of these cells to exposure to cisplatin; at high doses, the cells rapidly underwent apoptosis, whereas at lower doses the cells were blocked in G_2. After blocking in G_2, the cells could either regain their proliferating ability and re-enter the cell-cycle or could die from failure to overcome the G_2 arrest (Sorenson et al., 1990; Otto et al., 1996). When resistant cells from a human ovarian carcinoma cell line were compared to their sensitive, parental counterparts, it was found that the resistant cell lines needed higher doses of cisplatin to induce apoptosis (Fajac et al., 1996). Another study on ovarian cancer cells demonstrated that resistant cells overexpressed the protein product of the bcl-2 gene (Eliopoulos et al., 1995), which is a negative regulator of apoptosis. However, in another study, no correlation between cisplatin sensitivity and the ability to engage in apoptosis was observed (Ormerod et al., 1996). There are major differences in the intrinsic propensity of various cells to undergo apoptosis (Borner et al., 1995;

Fan et al., 1995), and cycling cells are far more vulnerable to cisplatin induced apoptosis than non cycling cells (Evans and Dive, 1993; Ormerod et al., 1996). Finally, mutations in genes involved in apoptosis, such as p53, can also play a role in the ability of cells to undergo apoptosis (Anthoney et al., 1996). Various attempts have been made in the last few years to correlate p53 status to chemosensitivity in clinical tumors. Specifically, as regards the susceptibility of ovarian cancers to cisplatin, two studies, based on a combined immunohistochemical and molecular analysis, suggested that tumors with p53 aberrations are significantly less sensitive to cisplatin-based chemotherapy than those with functional p53 (Buttitta et al., 1997; Righetti et al., 1996). In one of these studies (Righetti et al., 1996) the correlation between p53 alteration and response to chemotherapy was however only found for one specific type of mutation (missense) and p53 accumulation by immunohistochemistry was observed only in cases with this type of mutation. Nevertheless, in another series no relation between p53 alterations analysed using immunohistochemistry and PCR followed by DNA-sequencing, and response to cisplatin-based chemotherapy was observed (Di Leo et al., 1995). Similar results were found in another immunohistochemical study in stage III and IV patients (Jacquemier et al., 1994). In addition, no correlation between chemosensitivity and p53 accumulation was found in another group of patients analysed by immunohistochemistry combined with molecular analysis (Smith-Sorensen et al., 1998)

1.5 Circumventing cellular resistance to cisplatin

For many human tumours the clinical efficacy of the chemotherapeutic agents such as cisplatin, is still limited by different factors. These limitations have resulted in a great deal of effort having been expended to identify and/or develop new active anticancer molecules with higher antitumour activity and lower side effects.

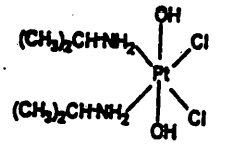
In recent years the molecular mechanisms by which cisplatin exerts its cytotoxic activity and those probably responsible for cellular resistance to cisplatin have been identified. The proposed mechanisms may contribute to development of a variable degree of cellular resistance but none of them completely explains the resistance of several tumours or the pattern of cross-resistance among different damaging agents.

These findings have stimulated intensive effort in the development of new platinum-based compounds with improved pharmacological properties. In this context, the most important strategy used is the modification of platinum-based preexisting molecules in order to develop non cross-resistant analogues of cisplatin. For example, cytotoxic activity of platinum-based drugs could be enhanced through the synthesis of derivatives that form DNA adducts with higher affinity for High Mobility Group (HMG)-proteins thus shielding these lesions from NER repair proteins more efficiently than the cisplatin DNA lesions. (McA'Nulty and Lippard, 1996a; Anin. and Leng, 1990).

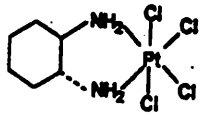
Investigations performed on the trans isomer of cisplatin (transplatin) evidenced that this molecule did not have any antitumoural activity because it is sterically unable to form the very toxic 1,2-GpG and 1,2-ApG intrastrand cross-links. The lack of activity of transplatin might be associated with the formation of high levels of monofunctional adducts mostly detoxified by glutathione through a rapid reaction whereas a minority probably rearranges to form bifunctional 1,3 and 1,4 GpG intrastrand cross-links or DNA interstrand cross-links likely removed by DNA repair systems.

A large number of mononuclear platinum compounds have been developed as potential candidates for clinical use (Fig. 15). Among these compounds, carboplatin [cis-diammine, 1,1-cyclobutane dicarboxylato platinum (II)] was the first additional platinum complex which received worldwide registration and acceptance, With carboplatin the major toxicity found for cisplatin have been substantially reduced (Calvert et al., 1985; Yarbo, 1992) although randomised studies showed that the two drugs appear broadly comparable in terms of response rates (Mangioni et al., 1989; Horwich et al., 1992). Furthermore, several studies documented a certain degree of cross-resistance between the two compounds probably due to the fact that the cellular and molecular mechanisms by which these drugs induce their cytotoxic activity are similar. Thus, while carboplatin unequivocally offers to patients a more acceptable level of morbidity compared to cisplatin, the clinically significant problems of intrinsic and acquired cisplatin resistance persist. These data thus indicated that carboplatin greatly alleviated the high level of patient morbidity

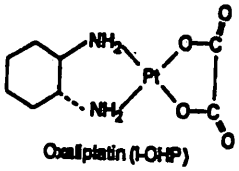
Figure 15. Chemical structures of some of mononuclear platinum-based agents used in clinical trials.



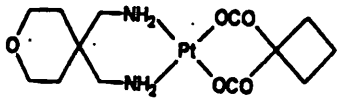
Iproplatin (CHIP, JM9)



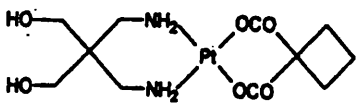
Tetraplatin (Ormaplatin)



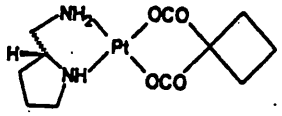
Oxaliplatin (l-OHP)



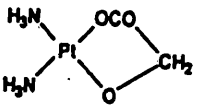
Enloplatin (CL287, 110)



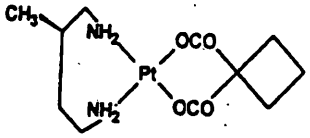
Zeniplatin (CL286, 558)



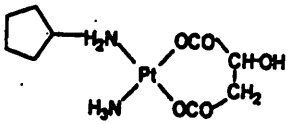
DWA2114R



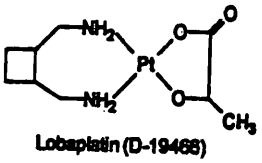
254-S



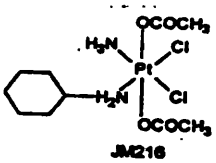
CI-973 (NK121)



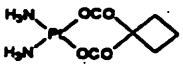
Cycloplatam



Lobaplatin (D-19466)



JM216



CARBOPLATIN

-Figure 15-

but it was also evident that the greatest problem remained the circumvention of the cellular resistance. This goal was accomplished by defining strategies based on refinements of preclinical tumour models for agent evaluation and rationally-driven synthetic chemistry through integration of recently accrued knowledge of tumour resistance mechanisms to both cisplatin and carboplatin.

For many years, preclinical antitumour evaluation of potential platinum-based anticancer drugs involved screening for activity against rapidly growing transplantable murine tumor cell lines, such as L1210 and P388 leukemias (Vendetti, 1983) and their variants possessing acquired resistance to cisplatin. Although this approach led to the discovery of platinum complexes 1,2-diamminocyclohexane (DACH), 1,2-diamminocycloheptane and tetraplatin, an important caveat concerning the adoption of these preclinal models was evident. In fact, DACH complex and tetraplatin were found to be very active against L1210 cells but inactive against cisplatin-resistant variant of the murine ADJ/PC6 model (Goddard et al., 1991).

To get round the problem described above, a different screening strategy was adopted. A panel of human ovarian cancer cell lines, containing examples of both intrinsic and acquired resistance to cisplatin and, where possible, well characterised for mechanisms underlying resistance/sensitivity to this drug, was selected.

By means of this innovative approach, several cisplatin-derived compounds chemically synthesised were evaluated for their antitumoural activity, general toxicity and cross-resistance with respect to cisplatin. Among these, the most

important and promising drugs are JM216 (bis acetate ammine dichlorocyclohexylamine platinum IV) and oxaliplatin (oxalate-1,2-diamminocyclohexane platinum II).

JM216 is an orally administered platinum compound which has been evaluated in phase II clinical trials against small-cell lung cancer, non-small-cell lung cancer, prostate and ovarian cancer and is currently undergoing phase III clinical evaluation (McKeage et al., 1995). It was selected as the lead compound of a series of ammine/amine Pt (IV) dicarboxylates, designed to show improved lipophilicity and gastrointestinal stability in an effort to overcome the limited gastrointestinal absorption observed with cisplatin and carboplatin (Giandomenico et al., 1991).

In preclinical studies, JM216 exhibited activity comparable to that of cisplatin against a panel of *in vitro* human ovarian carcinoma cells lines (Kelland et al., 1993a), lung cancer (Twentyman et al., 1992) and human cervical squamous cells (Mellish et al., 1993) and showed lack of cross-resistance to cisplatin in a panel of six pairs of human tumour cell lines, both parental sensitive and with acquired resistance to the latter drug. However, a very recent study (O'Neil et al., 1999) confirmed that JM216 exhibits a comparable level of cytotoxicity to that of cisplatin in human ovarian cancer cells sensitive (CH1) or with acquired (CH1cisR9) and intrinsic (SKOV-3) resistance to cisplatin, thus indicating a certain degree of cross-resistance between cisplatin and JM216 in these cell lines. Other studies found that this drug, similarly to other platinum-based drugs, is able to exert its cytotoxic

activity through induction of p53-dependent apoptosis and that cellular resistance could be attributable to an increased tolerance to platinum-DNA adducts, to an enhanced gene-specific repair and to elevated GSH levels.

Oxaliplatin is a member of the family of DACH platinum complexes and is a third generation platinum antitumour drug (Chaney, 1995). At present it has entered in clinical European phase II trials and data obtained thus far indicate an encouraging activity and manageable toxicity in a variety of tumours usually resistant to cisplatin (Cvitkovic, 1998).

As mentioned above, the advantages of oxaliplatin over cisplatin include less severe clinical toxicity and retained activity against cisplatin-refractory tumours (Kelland, 1993b; Kelland and McKeage, 1994). Preclinical studies performed on both *in vitro* and *in vivo* experimental models confirmed that this derivative exhibits different spectra of activity and toxicity from cisplatin, often with a lack of cross-resistance between the two agents (Tashiro et al., 1989; Pendyala. and Creaven, 1993; Chaney, 1995).

Comparative analysis of the sensitivity pattern assayed in a panel of 60 tumour cell lines suggested that oxaliplatin differs mechanistically from cisplatin and other platinum drugs (Rixe et al., 1996). In fact, it is likely that the trans-R,R configuration of the DACH moiety confers superior cytotoxic activity and drug binding to cellular DNA (Inagaki and Sewaki, 1995). Experimental results indicated that oxaliplatin exerts its cytotoxic effect through mechanisms of action and/or of resistance probably different from those of cisplatin (Woynarowski et al., 1998). In fact, investigations performed

on the human ovarian cancer cell line A2780 evidenced that lesions produced by oxaliplatin on cellular DNA are fewer than those of cisplatin. Furthermore, after exposure of cells to the two drugs, general platination levels found in oxaliplatin-treated cells are lower than those found in cisplatin-treated cells, thus indicating that to achieve comparable cytotoxicity fewer oxaliplatin DNA adducts are necessary.

It has also been hypothesised that this higher oxaliplatin cytotoxic activity could reflect a more difficult repair of DNA lesions with respect to those of cisplatin although, in different experimental models and using different experimental approaches, some workers evidenced a similar DNA-lesion removal capacity between the two drugs (Woynarowski et al., 2000). Very recent data (Woynarowski et al., 2000), tentatively explain the greater lethality of oxaliplatin in comparison to cisplatin by a reduced replicative bypass (thus, by an elevated inhibition of DNA chain elongation) and by a different processing of oxaliplatin-DNA lesions which could also elicit different downstream responses. For instance, cisplatin had been found maximally active in cells where the MMR DNA repair pathway was intact (Aebi et al., 1996; Fink et al., 1996) whereas oxaliplatin adducts are poorly recognised by MMR proteins (Fink et al., 1996) and the drug retains a high cytotoxic activity in MMR-deficient cells.

1.6 The novel multinuclear platinum complex BBR 3464

Recently, an alternative option in the design of platinum-based analogues has led to the development of multinuclear platinum compounds. Dinuclear and trinuclear platinum complexes represent a new class of anticancer agents distinct for DNA binding and antitumour activity from their mononuclear counterparts. Moreover, chemical studies have shown that dinuclear and trinuclear compounds represent a large and diverse class of structures which may be differentiated among themselves with respect to important parameters of biological activity (Farrell, 1996). In fact, antitumour activity of bifunctional dinuclear and trinuclear platinum complexes is affected by the nature of the coordination sphere, chain length and steric effects within the linker group (Farrell et al., 1999).

Dinuclear platinum complexes are a class of compounds of considerable interest for their antitumour and DNA-binding properties (Farrell, 1993) and the utility of the dinuclear motif in drug design was first demonstrated by linking two antitumour active cisplatin molecules by a flexible diamine chain (Farrel et al., 1988). Incorporation of the cisplatin synthon into linear trinuclear systems was also achieved (Brabec et al., 1999).

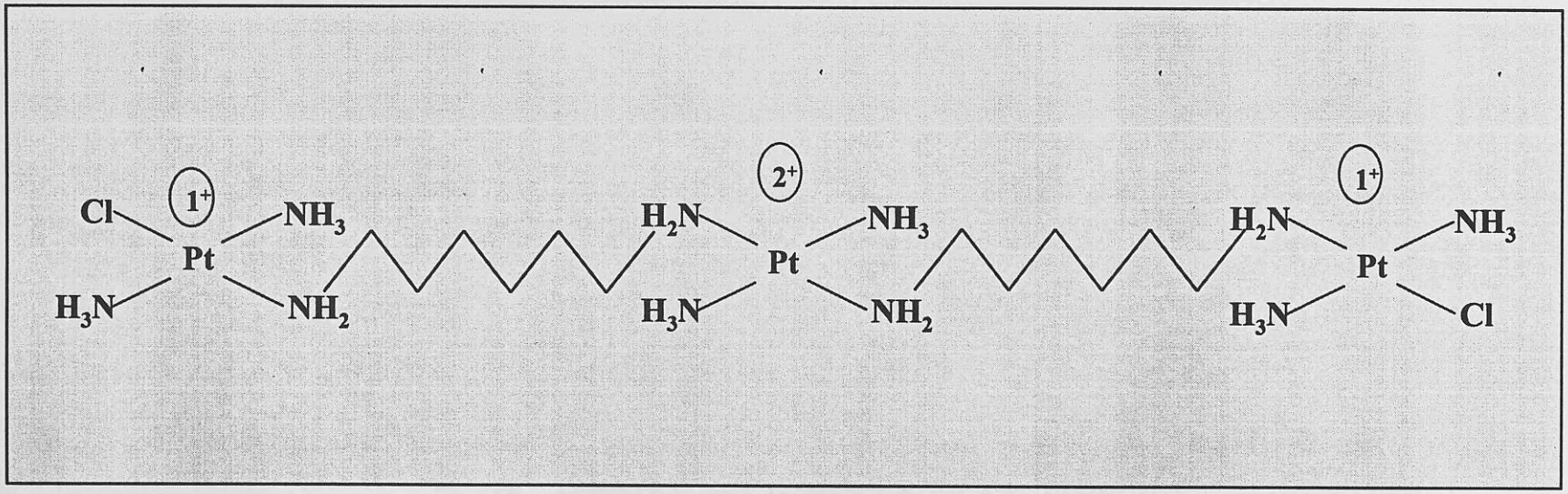
Although these molecules did not contain a cis-[$PtCl_2$(amine)n] unit, they showed the same cytotoxic and antitumoural effect of cisplatin when used at similar doses (Farrell, 1994). It has been demonstrated that the properties of monofunctional spheres and an overall 2^+ charge violate all previous structure-

activity relationships for platinum complexes. The complexes cause significant DNA (Pt,Pt) interstrand (Zou et al., 1994, Farrell. et al., 1990) as well as (Pt, Pt) intrastrand cross-links (Zou et al., 1994). Binding to DNA not only occurs at sequences preferred by cisplatin but other sequences, especially alternating purine-pyrimidine GCGC sequences, are attacked (Zou et al., 1994; Wu. et al., 1994; Farrell. et al., 1990). One of these compounds, [{trans-$PtCl(NH_3)_2\}_2H_2N(CH_2)_nNH_2]^{2+}$ also called BBR 3005, was also found to promote the B → Z conformational change of poly(dG-dC)•poly(dG-dC) at very low binding as evident from circular dichroism studies (Johnson et al., 1992). This finding is important because it has been demonstrated that DNA conformational changes such as unwinding and bending could alter the protein recognition of DNA. Particularly, it has been observed that the bending of DNA upon cisplatin binding is likely to be responsible for the recognition of platinum-lesions by HMG proteins which seems to be, as discussed above (DNA repair section), an important determinant for cisplatin cytotoxic activity (Lilley, 1992; Pil. and Lippard, 1992). In fact, a number of evidences suggest that HMG proteins recognise DNA damaged by BBR 3005 but not as efficiently as for cisplatin-damaged DNA. (Farrell et al., 1995), thus indicating that this molecule represents the first example of highly cytotoxic platinum-based agents which are not recognised by HMG proteins and suggesting that the nature of bis(platinum)-DNA adducts allows for a systematic design of a "HMG bypass" mechanism. Very interesting was the evidence that BBR 3005 displays cytotoxicity and antitumour activity in its own right and especially

retains activity in cell lines resistant to cisplatin (Farrell et al., 1990). Thus, the linking of two mononuclear, monofunctional antitumour inactive moieties produces formally bifunctional DNA binding agents. In this manner, chemical and biological comparison with the classical monuclear cis- and trans-$[PtCl_2(NH_3)_2]$ could be made (Farrell et al., 1995).

Most of the reported considerations are also valid for BBR 3464, the first representative of the trinuclear platinum complexes class. However, an enhanced antitumour activity was found for this drug in comparison to BBR 3005. BBR 3464, which has recently entered phase I clinical trials, is a novel anticancer agent designed on the hypothesis that new clinically useful platinum-based anticancer agents should have novel structures unrelated to those of agents currently used in the clinic. This trinuclear platinum complex is best described as two trans-$[PtCl(NH_3)_2]$ external units linked by a tetra-amine $[trans\text{-}Pt(NH_3)_2\{H_2N(CH_2)_6NH_2\}_2]^{2+}$ central unit (Fig. 16). The central unit is only able to form non-covalent- hydrogen bonding- interactions with DNA, whereas the two external units, contain chloride ions, when aquated, become potent positively charged electrophilic intermediates able to covalently interact with negatively charged-nucleophilic groups of DNA bases. These particular properties of the molecule improve its affinity for the DNA, affect the charge/lipophilicity balance and further increase the distance between the two Pt-DNA-binding coordinatination spheres, thus determining a mode of DNA binding different from that of any other clinically-used DNA-damaging agent.

Figure 16. Chemical structure of the novel trinuclear platinum complex BBR 3464.



-Figure 16-

An intriguing aspect of BBR 3464 is that, in adding charge and increasing chain length, interstrand cross-linking is diminished relative to BBR 3005 and long range intrastrand cross-links become equally probable. These types of lesions have critical consequences for the biological functions of DNA; the resulting conformational alterations in DNA might play an important role in considerably altering the antitumour effects of this new platinum complex as compared with cisplatin.

In preclinical studies performed by using different experimental models, BBR 3464 was found to be very active against human tumour cell lines characterised by intrinsic or induced resistance to cisplatin. Moreover, in these models an almost complete lack of cross-resistance between cisplatin and the novel trinuclear platinum complex was also observed (Pratesi et al., 1997; Giuliani et al., 1997; Manzotti et al., 2000). Further studies evidenced that BBR 3464 retains antitumoral activity in human tumor xenografts and human cancer cell lines carrying inactivating mutations in the p53 gene (Pratesi et al., 1999).

These data altogether suggest that this novel trinuclear platinum anticancer drug, which is characterised by high activity in a broad spectrum of human tumours commonly insensitive to platinum-based chemotherapeutic intervention, probably exerts its cytotoxic effect through molecular and cellular mechanisms different from those of cisplatin and cisplatin-derivatives. However, thus far, the cellular and molecular basis of BBR 3464 efficacy and

the relevance of factors involved in cisplatin resistance as determinants of response to the trinuclear platinum complex are still poorly understood.

1.7 Methods used to explore drug-DNA interactions

Although DNA interacting agents, currently used in clinical practice, are also toxic to normal tissues, they possess some degree of selectivity and specificity against a variety of human tumours. There are tumours which are particularly sensitive to DNA-interacting agents (for example testicular tumours are often curable with cisplatin), but the reasons for this peculiar sensitivity are still unclear. There is therefore a clear need for an arsenal of cellular, biochemical and biophysical techniques for characterising, with an highest approximation, how such compounds bind to DNA. In fact, a better understanding of the molecular basis of the sensitivity to such agents is necessary before any attempt can be made to design molecules which are more selective for different types of human neoplasm.

The rapidly growing knowledge of tumour biology, coupled with the use of novel molecular biology and pharmacology techniques, makes it possible to envisage experimental systems which could test precise hypotheses concerning the mechanisms of sensitivity and resistance to antineoplastic agents.

For several years, methods such as optical absorbance/fluorescence techniques, calorimetric assays, coupled mass spectroscopy analyses and electric dichroism-based procedures gave the possibility to investigate and

quantitate the overall DNA damage produced by DNA-damaging anticancer drugs both *in vitro* and in living cells. In the past, several studies performed to provide a better understanding of the chemical basis of the reaction by which DNA-damaging agents bind DNA, indicated that the major site of binding on DNA is the guanine N^7 position with lesser reaction at guanine O^6 and adenine N^3 .However, the above mentioned techniques do not give the possibility to directly confirm these chemical evidences, to ascertain whether a certain degree of sequence selectivity in DNA binding exists, to discriminate among the different types of DNA lesions or to evaluate if removal of such DNA adducts proceeds in a gene specific and/or strand specific manner.

1.7.1 Methods used to determine the sequence specificity of DNA-drug covalent binding

The availability of technical approaches to discriminate between the relative drug-DNA binding of a given base depending on the DNA sequence in which it was located can be very relevant, since one possible way to increase the specificity of DNA damaging agents could be to increase the damage towards selected DNA sequences which are important for the abnormal proliferation of cancer cells. Recently, methods for the determination of the pattern of covalent binding to guanine N^7 or adenine N^3 have been developed, thus making it possible to reevaluate existing DNA-damaging agents. The most widely used method is a modification of the Maxam-Gilbert DNA sequencing technique

based on the principle that the covalent binding of the drug to DNA can be converted to a strand cleavage either by hot piperidine in case of guanine N^7-drug adducts or by elevated temperature in the case of adenine N^3-drug lesions. The method has been successfully applied to obtain information on the pattern of guanine N^7 and adenine N^3 binding of several DNA-damaging agents used in cancer chemotherapy such as major groove alkylating agents (nitrogen mustards, chloroethylnitrosoureas and alkyltriazenes) and minor groove binders (tallimustine and CC-1065). However, this approach has important limitations in that it does not allow the determination of DNA-drug adducts which are not converted to DNA-strand breaks. For example, cisplatin lesions on guanine N^7 (and adenine N^7) as well as guanine O^6 alkylations produced by methylating agents MNU and MNNG cannot be pinpointed using these procedures as they are not converted to DNA-strand breaks after hot piperidine or elevated temperature treatment.

To overcome this technical limitation, several techniques have been developed and, at present, the sequence specificity of DNA covalent adducts produced by all DNA-damaging anticancer agents can be evaluated. Of particular relevance are footprinting assays. Initially developed to investigate the DNA sequence specificity binding of proteins to DNA, these assays were then adapted to characterise both the covalent and the non-covalent DNA binding of smaller molecules, including DNA-damaging anticancer agents *in vitro*. These techniques rely on the ability of ligands bound to DNA to protect DNA from the digestion of DNase I (Drew, 1984) or radical producing

chemicals such as methidiiumpropyl-Fe-EDTA (Dervan, 1986). The analysis of undigested DNA fragments by sequencing techniques gives information on the precise location and binding site of the ligand on the DNA.

In addition to footprinting approaches, the sequence selectivity of DNA covalent adducts can be evaluated using enzymes which are stopped by the presence of the adducts themselves. The analysis of DNA fragment produced by the termination of E. coli exonuclease III (Exo III footprinting; Royer-Pokora et al., 1981), bacteriophageal and bacterial RNA polymerases (monodirectional and bidirectional transcrptional footprinting; Philips and Cullinane, 1997) and E. coli DNA polymerase (polymerase inhibition assay; Dooley and Weiland, 1997), has been used to pinpoint DNA sequence-specific damage produced, *in vitro*, by several monofunctional alkylating agents (Weiland and Dooley, 1991, Broggini et al., 1995) and platinum (II) coordination complexes (Royer-Pokora et al., 1981).

The use of the thermostable Taq DNA polymerase instead of E. coli DNA polymerase further improved the polymerase inhibition assay, thus producing a method called Taq stop assay.

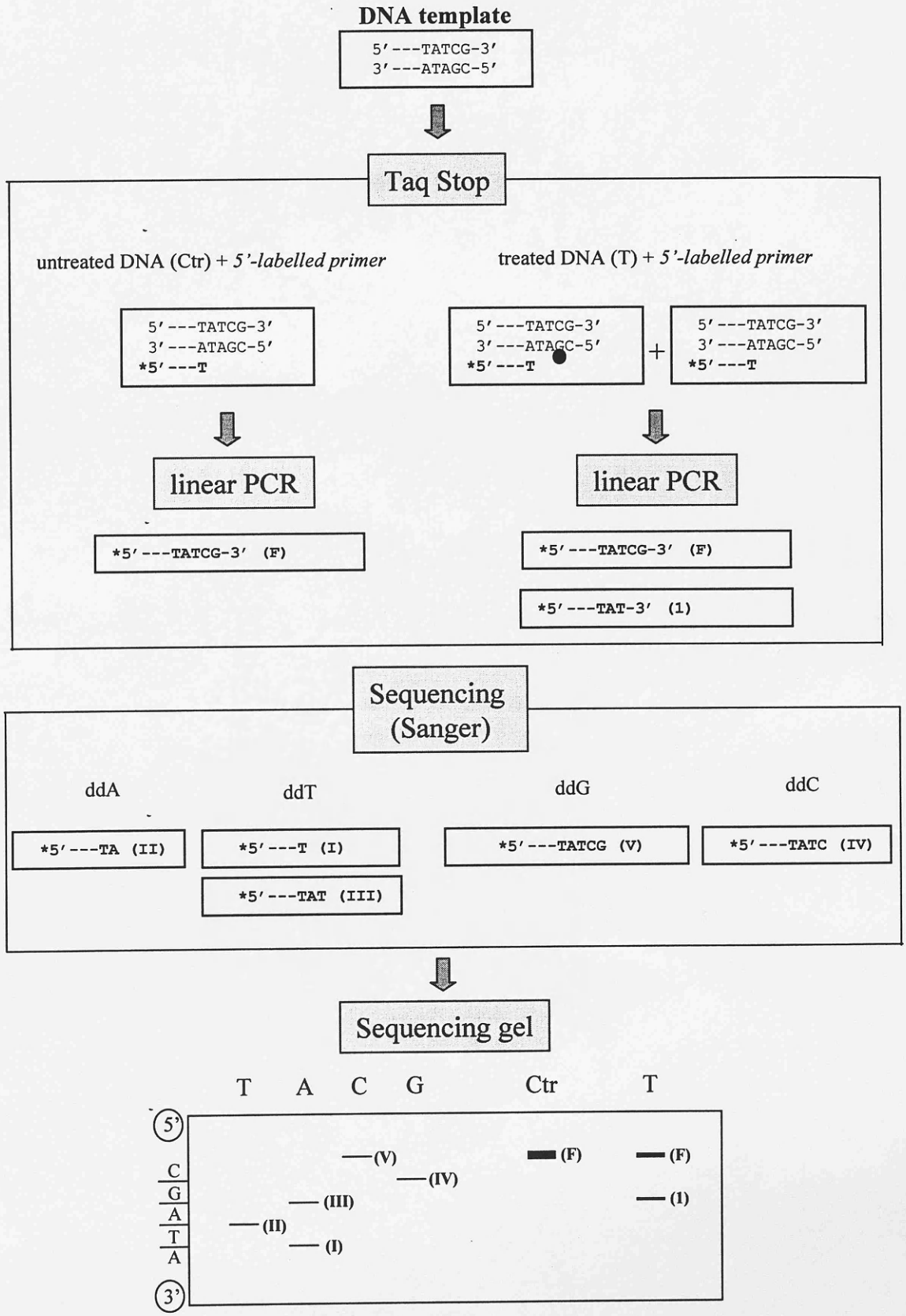
1.7.1.1 The Taq stop assay

The Taq stop assay (Ponti et al., 1991) is a linear amplification method employing the properties of DNA polymerase to investigate the sequence selectivity of the interaction between DNA-damaging agents and DNA. The

use of Taq DNA polymerase, instead of non-thermostable enzymes, gives the possibility to perform a number of polymerisation cycles (linear PCR) thus increasing the sensitivity of the system and, hence, allows detection of less frequent drug-DNA lesions. With respect to other methods, such as Exonuclease III, non-thermostable DNA polymerisation inhibition and chemical modification assays, this technique is characterised by higher simplicity, sensitivity, resolution power and sufficiently low background to allow the sequence specificity of DNA adducts to be easily determined both *in vitro* and in intact living cells. In addition, it is important to underline that this method is advantageous over other assays in that it is not limited to a single type of DNA damage: with the Taq stop approach, virtually all drug-induced DNA lesions are detectable.

As shown in Figure 17, an opportunely-selected oligonucleotide primer previously labelled is hybridised to a defined sequence of DNA and is extended by Taq DNA polymerase up to the drug-induced lesion. DNA-damaging agents produce adducts which, directly through steric obstruction or indirectly causing distortions in DNA, are able to inhibit polymerisation at the site of adduct formation. The forced blockage of the polymerisation caused by the lesions generates a pool of radioactively-labelled fragments the length of which is shorter than that of the full-length sequence and is determined by the position of DNA-drug adduct. Thus an analysis of the Taq stop reaction products on DNA sequencing gels allows the sequence specificity to be determined, with high precision (± 2 bp), by reference to the appropriate dideoxy sequencing

Figure 17. Schematic representation of the Taq stop assay. An opportunely-selected oligonucleotide primer previously labelled is hybridised to a defined sequence of DNA and is extended by Taq DNA polymerase up to the drug-induced lesion (★). The forced blockage of the polymerisation caused by the lesions generates a pool of radioactively-labelled fragments, the length of which is shorter than that of the full-length sequence and is determined by the position of DNA-drug adduct. By reference to the appropriate dideoxy sequencing lanes, the analysis of the Taq stop reaction products on DNA sequencing gels allows the sequence specificity to be determined with high precision.


DNA template
5′---TATCG-3′
3′---ATAGC-5′
Taq Stop
untreated DNA (Ctr) + 5'-labelled primer
treated DNA (T) + 5'-labelled primer
5′---TATCG-3′
3′---ATAGC-5′
*5′---T
+
linear PCR
linear PCR
*5′---TATCG-3′ (F)
*5′---TATCG-3′ (F)
*5′---TAT-3′ (1)
Sequencing (Sanger)
ddA
ddT
ddG
ddC
*5′---TA (II)
*5′---T (I)
*5′---TAT (III)
*5′---TATCG (V)
*5′---TATC (IV)
Sequencing gel
T
A
C
G
Ctr
T
5'
C
G
A
T
A
3'
(V)
(IV)
(III)
(II)
(I)
(F)
(F)
(1)

-Figure 17-

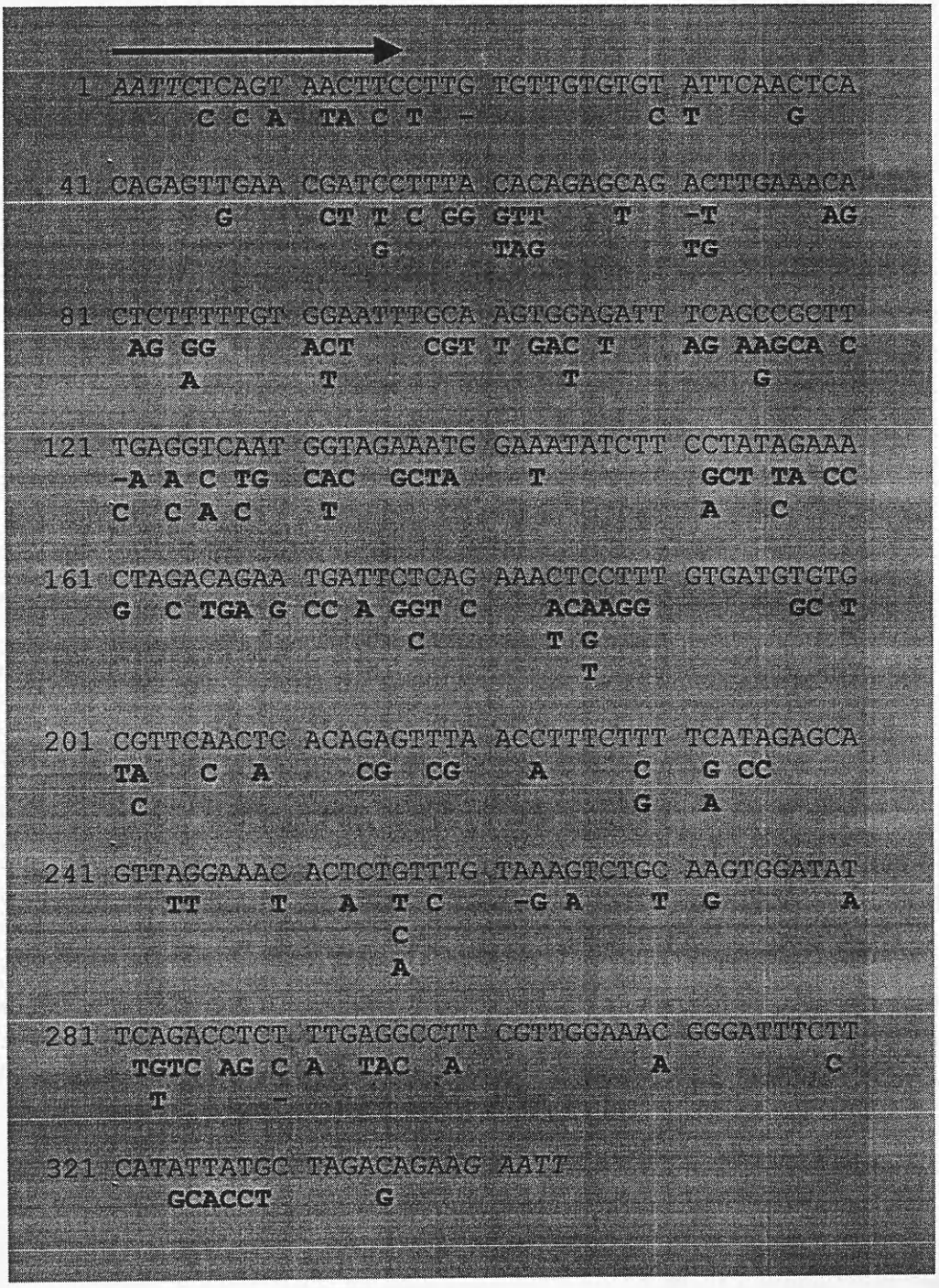
lanes. In addition, if the drug reactions employed produce single hit kinetics (each DNA molecule receives at most one lesion), the intensity of a band produced on the DNA sequencing gel is proportional to the extent of the covalent adduct at that base position.

The Taq stop assay has been widely used for examining the sequence selectivity of damage and repair in genomic DNA of living cells, an experimental condition significantly different to that of *in vitro* studies, in which deoxyribonucleic acid is associated with different proteins, packaged in higher ordered structures and in the nuclear compartment.

In many studies the DNA target investigated was α-RI DNA (Fig. 18), which is formed by approximately 100,000 copies of a 340 bp tandemly repeated sequence and represents about 1% of the overall genome (Murray et al., 1992) in human cells. Although the 340 bp repeat sequences are not perfectly homogeneous but contain a low level (7%) of random base substitutions, they are sufficiently homogeneous and in high copy number to allow the straightforward determination of the sequence specificity of DNA damaging agents. It is important to note that this genomic region does not have any function in the control of cellular metabolism, but it represents an optimal substrate in Taq stop assays in order to obtain a general picture of the sequence selectivity of a DNA damaging agent in a physiological situation.

Finally, results, which are more relevant from a physiological point of view, can be obtained determining the sequence specificity of anticancer drug-induced DNA lesions at single copy gene level within intact cells. This goal

Figure 18. The 340 bp α-RI DNA consensus sequence. In bold are indicated the possible base substitutions present in the different 340 bp α-RI DNA monomers compared to the consensus sequence. Dashes indicate possible deletions present in the different 340 bp α-RI DNA monomers compared to the consensus sequence. In italic are indicated the bases of the EcoRI sites. The underlined sequence indicates the position of the 16mer 'sense' primer (→).


1 AATTCTCAGT AACTTCCTTG TGTTGTGTGT ATTCAACTCA
C C A TA C T - C T G
41 CAGAGTTGAA CGATCCTTTA CACAGAGCAG ACTTGAAACA
G CT T C GG GTT T -T AG
G TAG TG
81 CTCTTTTTGT GGAATTTGCA AGTGGAGATT TCAGCCGCTT
AG GG ACT CGT T GAC T AG AAGCA C
A T T G
121 TGAGGTCAAT GGTAGAAATG GAAATATCTT CCTATAGAAA
-A A C TG CAC GCTA T GCT TA CC
C C A C T A C
161 CTAGACAGAA TGATTCTCAG AAACTCCTTT GTGATGTGTG
G C TGA G CC A GGT C ACAAGG GC T
C T G
T
201 CGTTCAACTC ACAGAGTTTA ACCTTTCTTT TCATAGAGCA
TA C A CG CG A C G CC
C G A
241 GTTAGGAAAC ACTCTGTTTG TAAAGTCTGC AAGTGGATAT
TT T A T C -G A T G A
C
A
281 TCAGACCTCT TTGAGGCCTT CGTTGGAAAC GGGATTTCTT
TGTC AG C A TAC A A C
T -
321 CATATTATGC TAGACAGAAG AATT
GCACCT G

-Figure 18-

was achieved by developing the single-strand ligation PCR in which an improved sensitivity was obtained by modifying the Taq stop assay (Grimaldi and Hartley, 1997).

1.7.2 Methods to evaluate induction and removal of drug-induced DNA lesions

A number of direct and indirect biophysical and biochemical techniques have been developed for the measurement of DNA interstrand cross-links induced by bifunctional DNA-damaging agents. Among these, the '*in vitro* cross-linking' and the alkaline elution assays are the most commonly used. By means of the first method ('*in vitro*' cross-linking assay) it is possible to measure the formation of interstrand cross-links after *in vitro* exposure of any defined DNA sequence to any DNA-damaging agent (Grimaldi and Hartley, 1997). The alkaline elution assay (Kohn et al., 1976.) is an important approach to evaluate the formation and repair of DNA lesions after exposure of living cells to anticancer drugs able to produce DNA-DNA interstrand cross-links and DNA-protein cross-links. The most important limitations of both these techniques are imposed by dealing with the inability to evidence the behaviour of DNA-lesions other than those produced by cross-linking agents and the inability to evaluate lesion removal in specific regions of the genome. These limitations are very important because a large category of DNA-damaging anticancer agents induce their cytotoxic effect as a consequence of their ability to form bulky

adducts rather than to produce cross-links. Moreover, for the rational design of new agents, characterised by novel sequence specificity (that may allow some degree of gene targeting), it is important to have methods that allow the sequence selectivity of binding to be studied in cells to see if the intended target sequence is being hit and to what extent individual lesions are repaired.

These problems have been overcome by using PCR-based methods exploiting the fact that covalent drug-DNA adducts can block Taq polymerase catalytic activity. By means of these PCR-based methods, all possible types of drug-DNA interactions can be studied both *in vitro* and in intact cells at various levels of resolution from gene regions (300-3000 bp; quantitative PCR) (Grimaldi et al., 1994a; Bingham et al., 1996) right down to the ultimate level of detection-individual nucleotides in single-copy genes in mammalian cells (Grimaldi. et al., 1994b).

1.7.2.1 Quantitative PCR

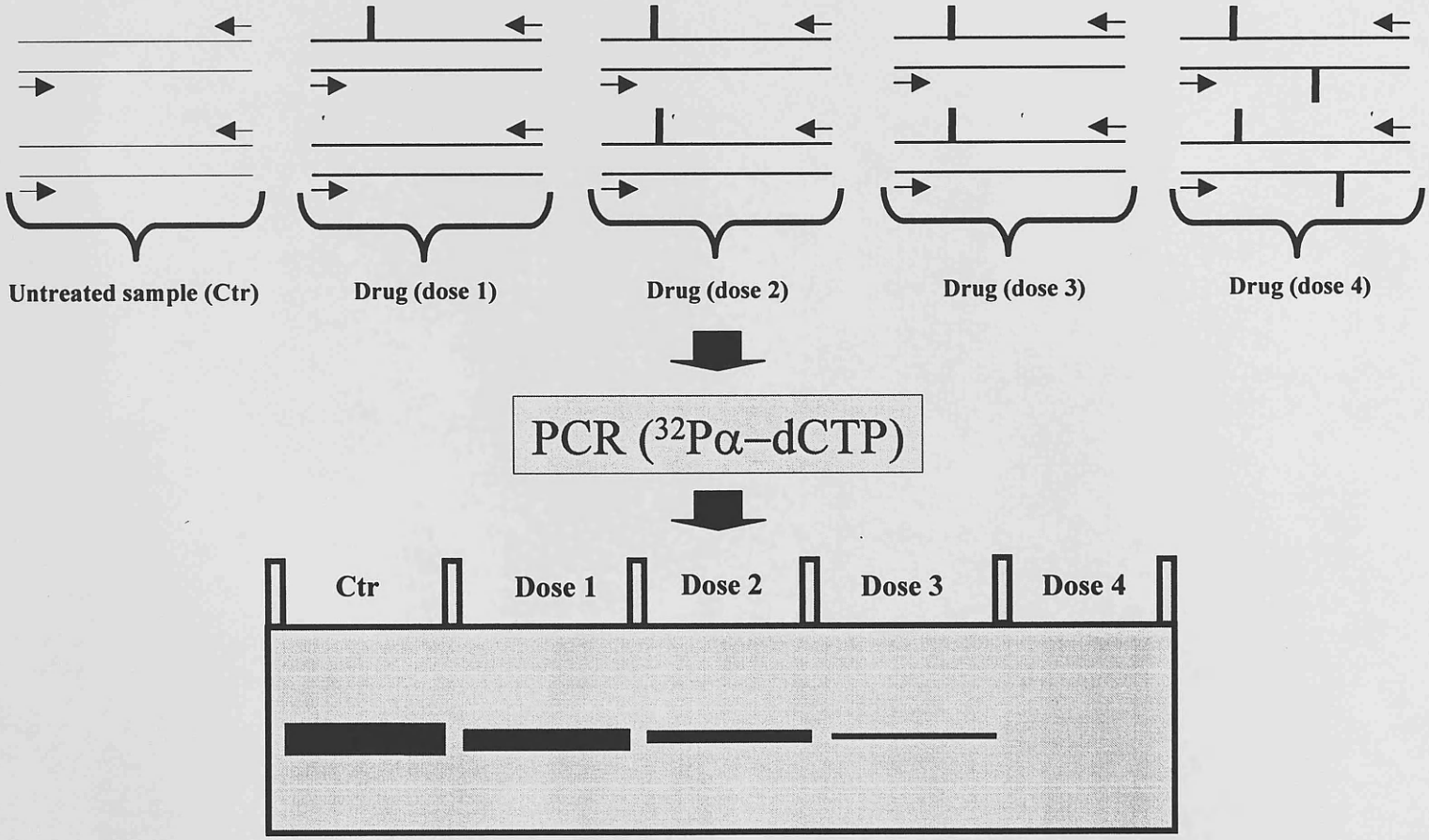
For many years the Southern blotting technique was used to study DNA damage and repair in living cells exposed to DNA-damaging agents not only in the overall genome but also in specific and physiologically relevant regions of the cellular DNA. However, an important limitation of this approach was the impossibility to perform such investigations at the subgene level. In view of the possibility that differential rates of induction and/or removal may occur both in different regions of a gene and in a strand-specific manner, the development of

a reliable quantitative and sensitive method for studying drug effects in small regions of a particular gene and/or in the two strands of the same gene region was necessary (Bingham et al., 1996).

PCR-based techniques recently developed are equally sensitive, more rapid and need less DNA compared with Southern blotting analysis. Moreover, PCR-based techniques, give the possibility to map DNA damage and repair at the subgene level. Among these assays, quantitative PCR is able to measure the aggregate damage on both strands in a gene region of choice. It is sensitive enough to be used to look at subgene functional regions such as introns, exons and promoters. Currently, a convenient size for amplification is between 300-3000 bp. However, with the availability of reagents allowing "long PCR" the upper limit may be extended up to 20-30 Kbp allowing quantitative PCR to be used to study entire genes, in order to compare, for example, the kinetics of DNA damage and removal in genes expressed at a different rate.

The principle of this technique is very simple. As shown in Figure 19, a pair of oligonucleotide primers, that define the region of the genome and/or the gene selected for the study, is used. In PCR each strand of DNA serves as potential template for exponential amplification and the presence of one or more adducts will block the amplification of that strand. Therefore, when drug-treated DNA is amplified and the reaction is stopped in the exponential phase, the amount of PCR product will be reduced compared to untreated DNA and the extent of the reduction will be proportional to the amount of damage caused by the drug treatment. Moreover, by including a radioactive nucleotide in the

Figure 19. Schematic representation of the quantitative PCR (QPCR) assay. An opportunely-selected pair of oligonucleotide primers is used to select and amplify a specific DNA sequence. In the PCR reaction each strand of such DNA sequence serves as potential template for exponential amplification and the presence of one or more adducts (I) will block the amplification of that strand. When drug-treated DNA is amplified and the reaction is stopped in the exponential phase, the amount of PCR product will be reduced compared to untreated DNA and the extent of the reduction will be proportional to the amount of damage caused by the drug treatment. The sensitivity of the assay can be increased by including a radioactive nucleotide in the PCR reaction so that the extent of damage caused by a specific treatment (and subsequent repair) can be accurately quantified.


Untreated sample (Ctr)
Drug (dose 1)
Drug (dose 2)
Drug (dose 3)
Drug (dose 4)
PCR (^{32}Pα–dCTP)
Ctr
Dose 1
Dose 2
Dose 3
Dose 4

-Figure 19-

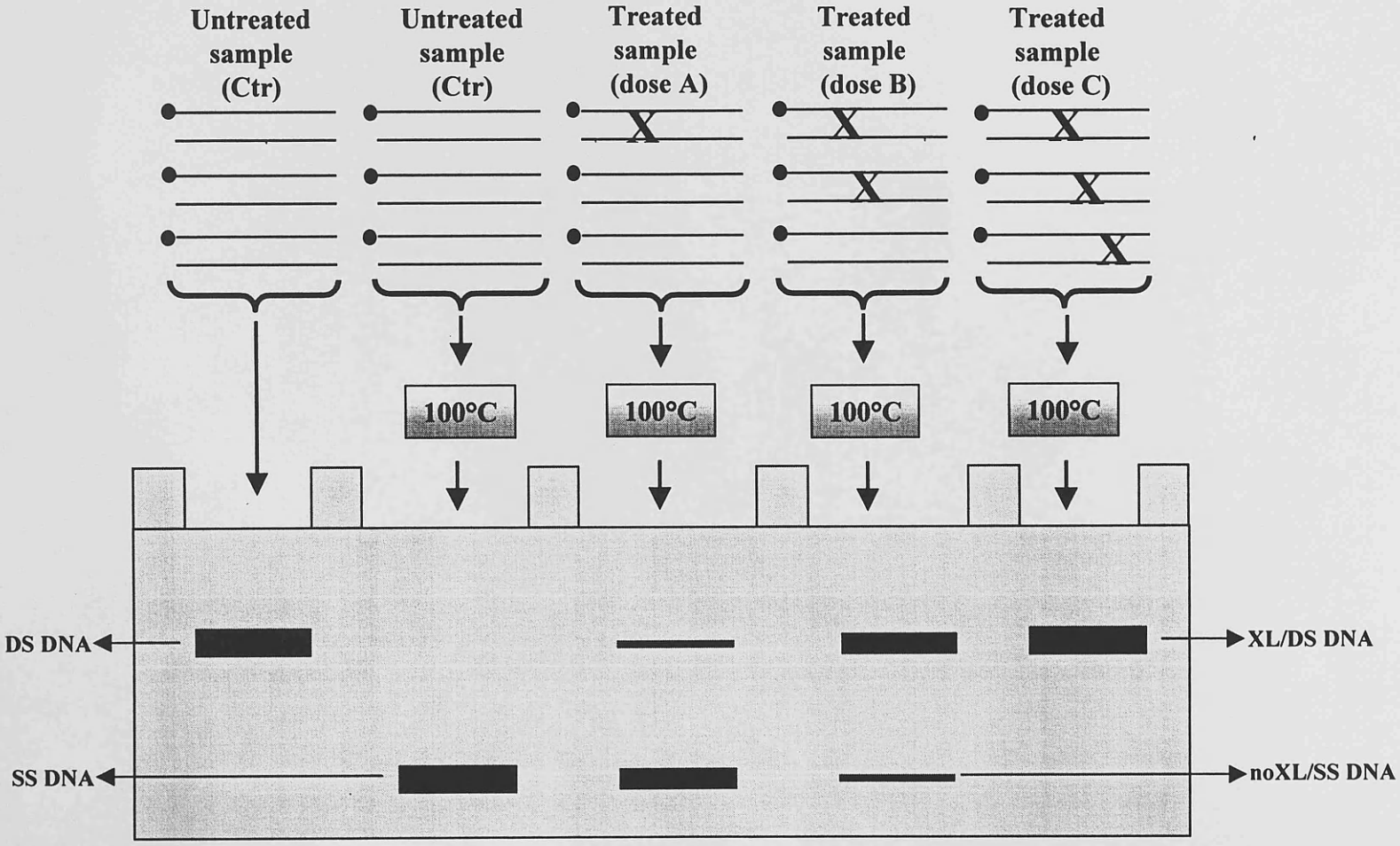
PCR reaction, the extent of damage caused by a specific treatment (and subsequent repair) can be accurately quantified.

1.7.2.2 The *in vitro* cross-linking assay

As mentioned above, the ability to produce interstrand cross-links on DNA could be an important molecular determinant for the antitumour activity of anticancer drugs. The possibility to rapidly compare the ability of a new DNA-damaging agent (for example BBR 3464) to produce interstrand cross-links *in vitro* with respect to a well known agent (for example cisplatin) could be an important tool to preliminary characterise its molecular and pharmacological properties.

The '*in vitro* cross-linking assay' gives the possibility to perform this screening step and allows the investigator to evaluate the kinetics of interstrand cross-links formation in an opportunely selected naked DNA sequence. As shown in Figure 20, a naked DNA sequence previously labelled and containing consensus sequences for the formation of interstrand cross-links, is exposed for an opportunely selected time to various concentrations of drug. Samples are then heat-denatured and loaded onto a non-denaturing agarose gel together with two untreated control samples, one of which was non heat-treated before loading. Thermal denaturation of the untreated control sample induces strand separation thus producing radioactive bands with higher electrophoretic mobility (single strand form) with respect to the corresponding non-heat

Figure 20. Schematic representation of the '*in vitro* cross-linking' assay. A naked DNA sequence previously labelled (★———) and containing consensus sequences for the formation of interstrand cross-links, is exposed for an opportunely selected time to various drug doses. Treated samples are then heat-denatured (100°C) and loaded onto a non-denaturing agarose gel together with two untreated control samples, one of which was non heat-treated before loading. Thermal denaturation of the untreated control sample induces strand separation thus producing radioactive bands with higher electrophoretic mobility (SS DNA) with respect to the corresponding non-heat denatured control sample (DS DNA). In the drug-treated samples, the formation of interstrand cross-links (X) partially inhibits strand separation thus producing an electrophoretic pattern in which double strand form band represents the DNA molecule pool in which the separation of the two complementary strands was inhibited by the presence of interstrand cross-links (XL/DS DNA) whereas single strand form band represents the DNA molecule pool in which strand separation was not inhibited by the interstrand cross-links (noXL/SS DNA). Moreover, the amount of interstrand cross-links present in the DNA molecules is proportional to the intensity of the double strand form band and/or to the decrease of the single strand form band.


Untreated sample (Ctr)
Untreated sample (Ctr)
Treated sample (dose A)
Treated sample (dose B)
Treated sample (dose C)
100°C
100°C
100°C
100°C
DS DNA
SS DNA
XL/DS DNA
noXL/SS DNA

-Figure 20-

denatured control sample (double strand form). In samples exposed to the drug, the formation of interstrand cross-links partially inhibits strand separation thus producing an electrophoretic pattern in which both single strand and double strand forms are present. In fact, the double strand form band represents the DNA molecule pool in which the separation of the two complementary strands was inhibited by the presence of interstrand cross-links. In addition, the amount of interstrand cross-links present in the DNA molecules is proportional to the intensity of the double strand form band and/or to the decrease of the single strand form band.

AIMS OF THE STUDY

(2)

Multinuclear platinum compounds represent a new approach to circumvent cellular resistance to cisplatin in that they are endowed with a different DNA-binding profile compared to their mononuclear counterparts. BBR 3464 has been identified as the most active member of this class of compounds. The drug is more potent than cisplatin and is active in cisplatin-resistant experimental human tumour models. However, the cellular determinants responsible for BBR 3464 activity are largely unknown. Moreover, the relevance of factors contributing to cisplatin resistance (such as decreased drug accumulation, increased detoxification, increased DNA repair and tolerance to DNA damage, and decreased cell susceptibility to undergo apoptosis) in determining the cellular sensitivity profile to BBR 3464 needs to be established.

The present study was undertaken to elucidate possible molecular mechanisms responsible for the antitumour activity of BBR 3464. To this purpose, the cytotoxic activity of the trinuclear platinum complex was evaluated in two pairs of human ovarian cancer cell lines sensitive and with experimentally induced resistance to cisplatin. One of the cisplatin-resistant cell lines proved to be more sensitive to BBR 3464 than the parental cell line and the other one was moderately cross-resistant to BBR 3464. In an attempt to identify the cellular characteristics and/or the molecular events responsible for the peculiar drug sensitivity profiles observed for the two drugs, we performed a detailed characterisation of the cellular models, in terms of ability to accumulate the drugs and expression and function of important DNA repair pathways such as MMR and NER. A detailed evaluation of the extent and type

of DNA binding by the drugs in terms of DNA platination, sequence preference of DNA adducts and kinetics of accumulation and removal of lesions at the single-gene (N-*ras*) level was also carried out in the different cell models.

Due to its ability to modify DNA in a way which is different from that of cisplatin, BBR 3464 could evoke different pathways of cellular response to DNA damage such as activation of cell cycle checkpoints and triggering of the apoptosis pathway, as a function of the genetic background of the tumour model. In this study experiments were carried out to verify whether the peculiar drug sensitivity profiles observed in our cellular models were attributable to a different effect exerted by cisplatin and BBR 3464 on cell cycle progression or to their different ability to activate the apoptotic pathway. As regards the effects on cell cycle, we investigated the disturbance exerted by drugs on the progression of cells throughout the different cycle phases as well as the interference with proteins that regulate G_2 to M transition. As far as programmed cell death was concerned, we determined whether or not each drug was able to affect the mitochondrial membrane potential ($\Delta\psi_{mt}$) and to activate caspases, as detected by the presence of degradation products of caspase-substrates such as lamin B.

MATERIALS AND METHODS (3)

3.1 GENERAL PROCEDURES

3.1.1 Drugs

Melphalan was obtained from Sigma (Milan, Italy). Cisplatin was obtained from Pharmacia-Upjohn (Milan, Italy). BBR 3464 was obtained from Novuspharma S.p.A. (Monza, Italy) as $(NO_3)^-$ salt. Valinomycin was purchased from Sigma. Teniposide was purchased from Bristol-M. Squibb as 10 mg/ml (15 mM) stock solution.

3.1.1.1 Melphalan preparation

Six hundred-fifty µl of a Perchloric Acid (70%; Carlo Erba Reagenti-Milan, Italy)/absolute ethanol (Carlo Erba Reagenti-Milan, Italy) solution [1:200 (v/v)] were added to 1.3 mg of powdered melphalan. The drug was completely dissolved by vortexing and successively diluted with Phosphate Buffered Saline (PBS) (Bio-Whittaker Europe-Verviers, Belgium) to a final concentration of 20 µg/ml. Melphalan stock solution (20 µg/ml) was stored as 1 ml aliquots at –20°C. At this temperature the drug proved to be stable for at least 7 months.

3.1.1.2 Cisplatin preparation

To obtain a 1 mg/ml (3.3 mM) cisplatin stock solution, 10 ml of PBS were added to cisplatin powdered stock and the drug was completely dissolved by vigorous vortexing immediately before use.

3.1.1.3 BBR 3464 preparation

One mg/ml (807.1 μM) BBR 3464 stock solution was prepared as follows: an opportune volume of PBS was added to the powder and the drug was dissolved by performing 3 ice-cold sonication steps at 55 watt (Branson Sonifier 250, Italscientifica-Genoa, Italy) for 15 seconds with 10 seconds of pause on ice between each step. The BBR 3464 stock solution was stored at –20°C as 100 μl aliquots: at this temperature, the drug solution was stable for at least two weeks.

3.1.1.4 Valinomycin preparation

To obtain a 1 mg/ml valinomycin stock solution, 10 ml of distilled water were added to 10 mg of powdered valinomycin and the drug was completely dissolved by vigorous vortexing immediately before use

3.1.2 Human tumour cell lines

OAW42 and OAW42Mer cell lines were kindly supplied by Dr. R.A. Britten (Department of Radiation Oncology, University of Liverpool, UK). OAW42 cell line was derived from the ascites of a patient with a papillary serous cystoadenocarcinoma of the ovary (Wilsonc, 1984). The OAW42Mer cell line, which is resistant to melphalan and cross-resistant to other alkylators including cisplatin, was obtained by exposure of the OAW42 cell line to increasing stepwise concentrations of melphalan (Britten et al., 1990).

Both A2780 and A2780cp8 cell lines were kindly supplied by Dr. R. Ozols (FoxChase Cancer Centre Philadelphia, PA, USA). A2780 cell line was derived from the ovarian carcinoma of an untreated patient. The corresponding cisplatin-resistant A2780cp8 subline, which survived intermittent exposure to 8 μM cisplatin, was obtained by exposure of the parental A2780 cell line to increasing stepwise concentrations of the drug (Behrens et al., 1987). The A2780d cell clone was selected in culture from A2780 cell line without drug exposure.

The colon carcinoma HCT-116 cell line was kindly supplied by Dr. M. Broggini (Department of Oncology, Istituto di Ricerche Farmacologiche "Mario Negri"-Milan, Italy).

LoVo, HeLa and HL-60 cell lines, derived from untreated patients with colon carcinoma, uterine cervix carcinoma and leukaemia respectively were purchased from American Type Culture Collection (Rockville, MD, USA).

3.1.2.1 Human tumour cell lines growth conditions

All cell lines were maintained as monolayers in 25-cm^2 flasks (Corning-Costar Italia-Milan, Italy) at 37°C in a 5% CO_2 humidified atmosphere in an air incubator (Mod. BBD 6220, Heraeus-Milan, Italy).

OAW42 and OAW42Mer cells were cultured in D-MEM/F12 medium, supplemented with 10% (v/v) Foetal Bovine Serum (FBS) (Bio-Whittaker Europe), 2 μM L-glutamine (Sigma), 0.25 units/ml insulin (Sigma) and 0.1%

(v/v) gentamycin (Schering-Plough-Milan, Italy) at a density of 2.5 x 10^5 cells per 25-cm^2 flask.

The culture medium for the melphalan-resistant OAW42Mer cell line was supplemented with 1 μg/ml of melphalan.

A2780, A2780cp8 and A2780 were maintained as monolayers in RPMI-1640 medium (Bio-Whittaker Europe), supplemented with 10% (v/v) FBS, 2 μM L-glutamine, 0.25 units/ml insulin, 100 units/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma) and 0.1% (v/v) gentamycin, at a density of 0.8 x 10^5 cells per 25-cm^2 flask.

HCT-116 cells were cultured using Iscove medium (Bio-Whittaker Europe), supplemented with 10% (v/v) FBS, and 0.1% (v/v) gentamycin, at a density of 0.8 x 10^5 cells per 25-cm^2 flask.

LoVo cell line was maintained in D-MEM/F12 medium, supplemented with 10% (v/v) FBS, 2 μM L-glutamine, 1x MEM vitamins 100x (Life Technologies Italia-San Giuliano Milanese, Italy) and 0.1% (v/v) gentamycin, at a density of 0.8 x 10^5 cells per 25-cm^2 flask.

HeLa cells were cultured using D-MEM/F12 medium, supplemented with 10% (v/v) FBS, 2 μM L-glutamine and 0.1% (v/v) gentamycin, at a density of 0.8 x 10^5 cells per 25-cm^2 flask.

HL-60 cell line was maintained as a monolayer at 37°C in a 5% CO_2 humidified atmosphere in an air incubator, using RPMI 1640 medium, supplemented with 10% (v/v) FBS, 2 μM L-glutamine and 0.1% (v/v) fungizone (Sigma), at a density of 0.8 x 10^5 cells per 25-cm^2 flask.

All tumour cells were cultured as follow: at set time points, specific growth medium was removed from the 25-cm^2 flask and the cellular monolayer was washed twice with 5 ml 37°C-heated PBS then, 1 ml of a Trypsin-EDTA solution (250 mg/litre EDTA, 500 mg/litre Trypsin 1:250) (Bio-Whittaker Europe) was added to each flask. After a 5 minute-incubation at 37°C with gentle shaking, cell detachment was checked under the microscope (Nikon TMS, Folabo-Buccinasco, Italy)and trypsin activity was blocked by addition of 5 ml of complete growth medium. Five ml-suspension containing detached cells was transferred in a sterile 15 ml conical tube (Falcon-Becton Dickinson Italia-Milan, Italy) and centrifuged at 1,500 rpm for 5 minutes at room temperature in a Varifuge 3.OR centrifuge (Heraeus Sepatech). Cell pellet was then carefully and extensively resuspended in 5 ml of growth medium and a small aliquot (100 µl) was transferred in a plastic 20 ml-vial (Falcon-Becton Dickinson) containing 10 ml of the isoton solution (azide-free balanced electrolyte solution; Coulter Scientific, Luton, UK). Single-cell suspension was checked under the microscope in a Burker camera, and the number of detached cells was calculated by counting in a particle counter (Coulter Counter, Coulter Scientific, Luton, UK).

Frozen stocks of human tumour cell lines were prepared as follows. Cells in logarithmic growth phase were detached from the flasks and counted as previously described. The percentage of viable cells was also determined by Trypan blue dye exclusion test by mixing, in a 0.5 ml clean conical tube (Eppendorf Italia-Milan, Italy) 5 µl of detached cells suspension, 45 µl of PBS and 50 µl of Trypan blue dye (Sigma). Five µl of Trypan blue dye-stained cell

suspension were placed onto a Burker camera and checked under the microscope. The number of viable cells generally exceeded 95%. After counting, the cell suspension was centrifuged at 1,500 rpm for 5 minutes at 4°C. The supernatant was removed and the cell pellet was carefully resuspended in 4°C-cold 'freezing medium' [D-MEM/F12 medium supplemented with 40% (v/v) FBS] at a density of 5 x 10^6 viable cells/ml. One ml of such a cellular suspension was then transferred in a 2 ml sterile Nalgene tube (Nalgene Company, Rochester, NY, USA) and placed on ice. One ml of cryoprotective medium [basal EAGLE'S medium with HANKS' BBSS and 15% (v/v) dimethylsulfoxide (DMSO)] (Bio-Whittaker Europe) was added to each tube and after a gentle resuspension, the tube was placed onto a Cryo 1°C Freezing Container (Nalgene) and stored at –80°C for the initial freezing step. After 16-24 hours, frozen cells were removed from the freezing container and stored under liquid nitrogen vapours in the Barnstead/Thermolyne containers (Barnstead/Thermolyne, Dubuque, IO, USA).

For cell thawing, frozen cells were incubated in a 37°C water bath (P.B.I International-Milan, Italy), transferred into a sterile 15 ml conical tube containing 10 ml of complete growth medium and centrifuged at 1,500 rpm for 5 minutes at room temperature. The cell pellet was then resuspended in 15 ml of growth medium and cells were seeded in a 25-cm^2 flask.

3.1.3 Nucleic acids quantification

Concentration and quality of solutions containing DNA, RNA or oligonucleotides were determined by spectrophotometric analysis as follows.

In two clean 1.5 ml tube (Eppendorf), a suitable volume of nucleic acid-containing solution (generally, 5 and 10 µl) was gently mixed with RNase- or DNase-free distilled water to a final volume of 200 µl to obtain a 1:40 and 1:20 dilutions. As the blank sample, a third clean tube containing 200 µl of RNase- or DNase-free distilled water was also prepared. The content of each tube was transferred into quartz cuvettes (Perkin-Elmer Italia-Monza, Italy), the cuvettes were then placed onto a Lambda 11/Bio spectrophotometer (Perkin-Elmer Italia and absorbance values at 260 nm (A_{260nm}) and 280 nm (A_{280nm}) of wave length were measured. The A_{260nm} and A_{280nm} values obtained with the blank sample were subtracted from the absorbance values obtained with the nucleic acid-containing samples, and DNA, RNA or oligonucleotide concentration was then calculated using these formulae:

µg/ml of DNA = (A_{260nm}) x (dilution factor) x (50 µg/ml);

µg/ml of RNA = (A_{260nm}) x (dilution factor) x (40 µg/ml);

µg/ml of oligonucleotide = (A_{260nm}) x (dilution factor) x (30 µg/ml);

The nucleic acid concentration was expressed as the mean of the values (µg/ml) obtained in the two independently-prepared and –measured dilutions.

The quality of nucleic acid solutions was evaluated by calculating the A_{260nm}/A_{280nm} ratio. It is well known that for both DNA and oligonucleotide, a ratio of 1.8-2 was indicative of a good-quality preparation, whereas high quality RNA solutions are characterised by a A_{260nm}/A_{280nm} ratio of 2.

3.1.4 Protein concentration determination

Protein concentration was determined according to Bradford (Bradford, 1976).

3.1.4.1 Calibration curve preparation

Solutions of bovine serum albumin (BSA) (1 μg/200 μl, 2 μg/200 μl, 4 μg/200 μl, 5 μg/200 μl, 10 μg/200 μl and 20 μg/200 μl) were prepared by dissolving powdered BSA (Sigma) in distilled water. In a 1.5 ml tube, 200 μl of each BSA solution were mixed with 600 μl of distilled water and 200 μl of Biorad Protein assay dye (Biorad-Milan, Italy). In the blank sample, 800 μl of distilled water were mixed with 200 μl of Biorad Protein assay dye. Samples were rapidly transferred into disposable cuvettes (P.B.I. International) and the absorbance at 595 nm wave length of each calibration point was measured in the spectrophotometer. The absorbance value corresponding the blank sample was then subtracted from the values obtained in the BSA-containing samples. Each calibration sample was run in triplicate. The calibration curve obtained in such a way, gave the possibility to extrapolate the exact absorbance value corresponding to a 1 μg of proteins present in the solution.

3.1.4.2 Protein concentration quantification

Concentration of extracted proteins was determined by mixing in a 1.5 ml tube 1 µl of protein extracts with 200 µl of Biorad Protein assay dye and distilled water to a final volume of 1 ml. Samples were rapidly transferred into disposable cuvettes and the absorbance at 595 nm wave length was measured in the spectrophotometer. By dividing the obtained absorbance value for that corresponding to 1 µg of proteins (obtained from the calibration curve), it was possible to calculate the protein concentration of each sample. Also in this case, each sample was run in triplicate.

3.1.5 Agarose gel preparation

All of the agarose gel electrophoresis experiments were carried out using Hoefer horizontal electrophoresis units (Amersham-Pharmacia Biotech Italia-Cologno Monzese, Italy).

Gels of different sizes and percentages (w/v = grams of powdered agarose per 100 ml of running buffer) were prepared in clean glass bottles by mixing suitable amounts of powdered agarose (Sigma) in 1x TAE [0.04 M Tris-Acetate (Sigma), 0.001 M EDTA (Life Technologies)] or 1x TBE [0.09 M Tris-Borate (Sigma), 0.002 M EDTA] running buffers. Agarose was dissolved by heating in a microwave cooker (Philips mod. 810; Philips Italia-Milan, Italy), cooled to about 50°C, transferred onto the casting tray and, after the insertion of a clean comb, left to polymerise at room temperature. When polymerisation was completed, the comb was removed and solidified agarose gel was transferred into the running tray and submerged with running buffer.

3.1.6 SDS-polyacrylamide gel preparation

All of the SDS-polyacrylamide gels used for protein separation (16 centimetres high/16 centimetres length/1.5 millimetres thick) were prepared as described by Laemmli et. al. (1970) and run in a Hoefer SE 600 vertical electrophoresis slab unit (Amersham-Pharmacia Biotech Italia).

Resolving gel (30 ml) was prepared in a 50 ml conical tube (Falcon- Becton Dickinson) by mixing a suitable volume of 40% (w/v) polyacrylamide stock solution (acrylamide:N,N'-methylenebisacrylamide molar ratio = 29:1) (Sigma) with 0.3 ml of 10% (w/v) SDS solution (Sigma) [final concentration = 0.1% (w/v)], 7.5 ml of 1.5 M Tris-HCl pH 8.8 solution (Sigma) (final concentration = 375 mM), 300 µl of 10% (w/v) ammonium persulfate (Sigma) [final concentration = 0.1% (w/v)] and 18 µl of TEMED (Sigma) [final concentration = 0.06% (v/v)], and subsequently poured into the gap between glass plates (previously washed with absolute ethanol) leaving sufficient space for the stacking gel (the length of the teeth of the comb plus 1 centimetre: about 3 centimetres). The poured resolving polyacrylamide gel solution was immediately and carefully overlaid with 0.1% (w/v) SDS solution (the overlay prevents oxygen from diffusing into the gel and inhibiting polymerisation) and the gel was placed in a vertical position at room temperature to polymerise. When polymerisation was complete (within 30-45 minutes), the overlay was poured off and the top of the gel was carefully washed several times with distilled water to remove any unpolymerised acrylamide. The stacking gel (10 ml) containing 5% (w/v) polyacrylamide (acrylamide:N,N'-

methylenebisacrylamide molar ratio = 29:1), 0.1% (w/v) SDS, 125 mM Tris-HCl pH 6.8, 0.1% (w/v) ammonium persulfate and 0.1% (v/v) TEMED was prepared in a 15 ml conical tube and poured directly onto the surface of the polymerised resolving gel. A clean comb was immediately inserted into the stacking gel solution and the gel was placed in vertical position to polymerise. When polymerisation was complete, the comb was removed, the wells were washed with distilled water to remove any unpolymerised acrylamide and the gel was mounted in the electrophoresis apparatus containing TGS running buffer [25 mM Tris base, 250 mM Glycine (Sigma), 0.1% (w/v) SDS, pH 8.3] in both upper and lower buffer reservoirs.

3.1.7 Sequencing gel preparation

The sequencing gel (50 centimetres length/40 centimetres wide/0.4 millimetres thick) was prepared and run into a S3S sequencing unit (Owl Scientific Inc., Woburn, MA, USA). Before their assembly, glass plates were cleaned by extensive washes with distilled water and absolute ethanol to avoid the formation of air bubbles when the gel was poured. To prevent the gel from sticking tightly to both plates and reduce the possibility that the gel tears when it is removed from the mold, the surface of the smallest glass plate which will contact the gel was treated with Gel Slick silicone solution (J.T. Baker, Phillipsburg, NJ, USA) according to the manufacturer's recommendations.

Sequencing polyacrylamide gel was prepared in a glass beaker by mixing polyacrylamide (acrylamide:N,N-methylenbisacrylamide molar ratio = 19:1)

(Sigma), powdered urea (Sigma), 10x TBE buffer (0.9 M Tris-Borate, 0.02 M EDTA) and distilled water in order to achieve, in a final volume of 90 ml, a 6% (w/v) polyacrylamide/7 M urea/1x TBE solution. To completely dissolve powdered urea, the mixture was incubated several minutes in a heated water-bath with occasional agitation, and polyacrylamide solution was then filtered through a 0.2 μm filter membrane. After addition of ammonium persulfate and TEMED [final concentrations of 0.05% (w/v) and 0.02% (v/v) respectively], polyacrylamide solution was poured into the gap between the glass plates. The flat side of the shark's tooth comb was immediately inserted for approximately 0.5 centimetres into the gel solution and clamped by using bulldog binder clips. The gel was placed in the horizontal position and allowed to polymerise over night at room temperature. Before the gel mold was transferred to the electrophoretic unit containing 1x TBE in the upper and lower reservoirs, the shark's tooth comb was carefully removed from the top of the gel, the flat loading surface of the gel was washed with 1x TBE to remove any fragments of polyacrylamide and a cleaned shark's tooth comb was reinserted with its teeth just sticking into the loading surface of the gel. The sequencing gel was heated to about 60°C before sample loading by means of a pre-run at constant power of 57 watt by using an Alex 300 power supply (M-Medical-Florence, Italy).

3.1.8 Non-denaturing polyacrylamide gel preparation

Non-denaturing polyacrylamide gels (16 centimetres high/16 centimetres length/1.5 millimetres thick) were prepared and run in a Hoefer SE 600 vertical electrophoresis slab unit (Amersham-Pharmacia Biotech Italia).

Polyacrylamide gel solution (30 ml) containing 5% (w/v) polyacrylamide (acrylamide : N,N'-methylenebisacrylamide molar ratio = 29:1), 1x TBE buffer, 0.07% (w/v) ammonium persulfate and 0.035% (v/v) TEMED was prepared in a 50 ml conical tube and subsequently poured into the gap between glass plates (previously washed with absolute ethanol). A clean comb was immediately inserted and the gel was placed in vertical position to polymerise. When polymerisation was completed, the comb was removed, the wells were washed with 1x TBE buffer to remove any unpolymerised acrylamide and the gel was mounted in the electrophoresis apparatus containing 1x TBE running buffer in both upper and lower reservoirs.

3.1.9 Preparation of the pSP73 vector-containing E. coli library

In order to obtain a permanent and stable source of pSP73 vector, the following procedures were performed.

3.1.9.1 Preparation of competent JM 109 E. coli cells for transformation

Fifty µl of sterile JM 109 E. coli cellular suspension (Promega Italia-Milan, Italy) were mixed, in a sterile 50 ml conical tube, with 10 ml of sterile LB medium [1% (w/v) Bacto-Tryptone (Life Technologies Italia), 0.5% (w/v)

Bacto-Yeast extract (Life Technologies Italia), 1% (w/v) NaCl (Sigma), pH 7.0] and allowed to grow in a 37°C-heated shaking incubator (GFL3031; Folabo) at 225 rpm over night. One ml of such liquid bacterial culture was then transferred into a sterile 500 ml bottle containing 100 ml of sterile LB medium and the bottle was placed into the 37°C-heated shaking incubator at 225 rpm.

To harvest bacterial cells in logarithmic growth phase, 2 hours later, 1 ml of liquid culture was transferred, under a laminar flow (Bluebeam 4; Arredi Tecnici Villa-Caronno Pertusella, Italy), in a disposable cuvette and the absorbance at 600 nm of wave length was read on the spectrophotometer. E. coli concentration in the liquid culture was calculated by considering that 1 A_{600nm} unit corresponds to about 8 x 10^6 bacterial cells/ml. When A_{600nm} of the liquid culture reached 0.3 units (corresponding to 240 x 10^6 cells/ml; 24 x 10^9 cells in 100 ml) the bacterial suspension was transferred, under a laminar flow, in two ice-cold sterile 50 ml conical tubes and the cell growth was stopped by placing the tubes on ice for 15 minutes. E. coli cells were pelleted by a 3,000 rpm centrifugation at 4°C for 10 minutes and, after the removal of LB medium, cell pellets were pooled in the same tube by gentle resuspension in 10 ml of ice-cold sterile 0.1 M $CaCl_2$ solution (Sigma). After addition of 40 ml of ice-cold sterile 0.1 M $CaCl_2$ solution, bacterial suspension was incubated on ice for 30 minutes and subsequently centrifuged at 3,000 rpm for 10 minutes at 4°C. The supernatant was then removed and the cell pellet was carefully resuspended in 5 ml of an ice-cold sterile 0.1 M $CaCl_2$-15% (v/v) glycerol (Sigma) solution. Such a bacterial suspension was incubated on ice for 24 hours. At the end of incubation, the bacterial suspension was mixed by gentle

pipetting, divided in 500 µl-aliquots (4.8 x 10^9 cells/ml; 2.4 x 10^9 cells in each 500 µl-aliquot) in sterile 0.5 ml Nalgene tubes, frozen by snap freezing in liquid nitrogen and stored at –80°C.

Fifty µl (240 x 10^6 cells) of freshly-prepared competent cells were transformed with 50 ng of a DNA vector able to confer ampicillin resistance and 1/100,000 (dilution factor, 10^5) of the bacterial suspension was plated as described in the 2.1.9.2 section. Transformation efficiency, calculated on the basis of the formula [(NUMBER OF COLONIES) x (10^3) x (DILUTION FACTOR) / 50 ng], was expressed as the "number of colony forming units (CFU) per µg of plasmid DNA". Generally, 10^6-10^8 CFU per µg of plasmid DNA were indicative of a good preparation of competent bacterial cells.

3.1.9.2 Transformation of bacteria

In an ice-cold 10 ml Falcon 2059 tube (Falcon-Becton Dickinson), 240 x 10^6 of competent bacterial cells (50 µl; section 2.9.1) were diluted to a final volume of 100 µl with 0.1 M $CaCl_2$ and 50 ng (5 µl) of purified pSP73 vector were then added. The mixture was gently mixed by tapping and the tube was chilled on ice for 30 minutes, incubated for 90 seconds at 42°C in a water bath and for 2 minutes on ice. After a 5-minute incubation at room temperature, 900 µl of LB medium were added and tube was placed into a 37°C-heated shaking incubator at 225 rpm for 1.5 hours. The tube was then centrifuged at 3,000 rpm for 5 minutes at room temperature, 950 µl of supernatant LB were removed

and bacterial cell pellet was resuspended in the remaining 50 µl, plated onto 90-mm dish (Corning-Costar Italia-Milan, Italy) containing selective solid LB medium [liquid LB medium, 1.5% (w/v) agar (Life Technologies), 50 µg/ml ampicillin (Mead-Johnson-Rome, Italy)] and incubated over night at 37°C. Each colony, representing ampicillin-resistant/transformed growing bacterial cells, was picked-up with a sterile disposable loop, dissolved in a 50 ml conical tube containing 10 ml of liquid LB medium supplemented with ampicillin (50 µg/ml final concentration) and allowed to grow over night at 37°C with 225 rpm shaking.

3.1.9.3 Small scale preparation of plasmid DNA ("miniprep")

To confirm the presence of pSP73 plasmid in ampicillin-resistant liquid culture, 8 ml of the bacterial suspension were used for "miniprep" by using Wizard Plus SV Minipreps Kit (Promega Italia) according to the manufacturer's instructions. Plasmid DNA concentration was determined spectrophotometrically as described in the section 3.1.3.

Electrophoretic analysis was performed on 1% (w/v) agarose mini gel prepared and run in 1x TAE buffer. About 500 ng of extracted and original DNA vectors were loaded and separated at 90 volt (SE300 power supply; Amersham-Pharmacia Biotech.) at room temperature for 1 hour in the presence of 500 ng of λ-BsteII molecular weight marker (Sigma). DNA fragments were visualised by placing the gel onto the UV-transilluminator (260 nm wave length) (Biorad) after a 30-minute staining with 1µg/ml ethidium bromide (Life

Technologies Italia)-1x TAE buffer staining and a 30-minute 1x TAE buffer destaining.

3.1.9.4 Storage of pSP73 vector-transformed bacteria

In a 2 ml sterile Nalgene tube, 1.6 ml of ampicillin-resistant liquid bacterial culture and 0.4 ml of sterile pure glycerol (final concentration (v/v) = 20%) were mixed by a vortex mixer and frozen by snap freezing in liquid nitrogen and stored at –80°C.

3.1.10 Large scale preparation of plasmid DNA ("maxiprep")

By means of a sterile spatula, frozen bacterial cells were scraped from the 2 ml sterile Nalgene tube and dissolved in 10 ml of 50 μg/ml ampicillin containing-LB medium (contained in a 50 ml conical tube) and allowed to grow at 37°C in a shaking incubator at 225 rpm. After 8 hours, 10 ml liquid culture was mixed with 100 ml of fresh 50 μg/ml ampicillin containing-LB medium into a 500 ml glass bottle and cells were left to grow over night at 37°C and 225 rpm.

Bacterial cells were pelleted by a 30-minute centrifugation at 4,000 rpm at 4°C and, after removal of LB medium, plasmid DNA was purified with the Qiagen Plasmid Maxi Kit (Qiagen-Hilden, Germany) according to the manufacturer's instructions.

Concentration and quality of the plasmid DNA were evaluated as described in the section 3.1.3.

3.1 SPECIFIC PROCEDURES

3.2.1 Cytotoxic activity of cisplatin and BBR 3464 on cell lines: the growth inhibition assay

After harvesting in logarithmic growth phase, cells were seeded in 6-well plates (Corning-Costar Italia) in 2 ml of fresh media at the following densities: 5 x 10^4 cells/well for OAW42 and OAW42Mer and 4 x 10^4 cells/well for A2780, A2780d and A2780cp8. The plates were then incubated at 37°C in a 5% CO_2 humidified atmosphere in the air incubator for 24 hours. As regards the OAW42Mer cell line, it is important to stress that melphalan was not added to the growth medium for at least 2 passages before initiation of the growth inhibition experiment.

Immediately before cell treatment, cisplatin (3.3 mM) and BBR 3464 (807.1 μM) stock solution were diluted in specific growth media to obtain the different 11x working solutions of the two drugs. Two hundred μl of each drug 11x working solution were added to each well (containing attached cells and 2 ml of medium) in order to expose the cells to 1x final drug concentration in a final volume of 2.2 ml. For untreated control samples, 200 μl of fresh medium was added to each well. After a 1-hour incubation of cultures at 37°C in a 5% CO_2 humidified atmosphere in air incubator, drug-containing medium was

removed from each well and cells were washed three times with PBS (3 ml for each well). Five ml of fresh medium were then added to each well and plates were incubated at 37°C in a 5% CO_2 humidified atmosphere in air incubator. After 72 hours, medium was removed from each well, cells were washed three times with PBS (3 ml for each well) and detached from the plates by a 5-minute incubation at 37°C with Trypsin-EDTA solution (1 ml for each well). Trypsin activity was stopped by addition of a 10% (v/v) FBS-PBS solution (5 ml for each well) and cells were transferred in the isoton solution. Single-cell suspensions were obtained by repeated pipetting, checked under the microscope and counted in a particle counter.

Each experimental sample was run in triplicate and the results were expressed as the number of cells in treated samples compared with control samples. Each experiment was repeated at least three times.

3.2.2 Expression of mismatch repair (MMR) and nucleotide excision repair (NER) proteins

Basal level expression of some of the most important proteins involved in MMR and NER DNA repair pathways were determined in OAW42, OAW42Mer, A2780, A2780d and A2780cp8 cell lines by Western blot analysis.

3.2.2.1 Nuclear extracts preparation

Proteins were extracted from cell nuclei by using a modification of the method described by Dignam (Dignam et al., 1983).

OAW42, OAW42Mer, A2780, A2780d, A2780cp8, HCT-116, LoVo and HeLa cell lines in logarithmic growth phase were detached from the flasks by means of Trypsin-EDTA solution, transferred in a 15 ml conical tube and pelleted by a 5-minute centrifugation at 1,500 rpm at 4°C. To obtain a single-cell suspension, cell pellets were dissolved by repeated pipetting in 10 ml of PBS. Cell suspension was checked under the microscope, and a small aliquot (100 µl) was transferred in the isoton solution and counted in the particle counter.

An aliquot of cell suspension containing about 5 x 10^6 cells was then transferred in a 1.5 ml conical tube and cells were pelleted by a 15-second centrifugation at 12,000 rpm at 4°C (5417 R Centrifuge) (Eppendorf Italia). After removal of PBS, cell pellets were again washed twice with PBS. At the end of the last wash, PBS was removed and, in order to destroy the cellular membrane but not the nuclear membrane, cells were resuspended by gentle pipetting in 400 µl of ice-cold/slightly hypotonic lysis buffer A [10 mM Hepes pH 7.9 (Sigma), 10 mM KCl (Sigma), 0.1 mM EDTA pH 8, 0.1 mM EGTA (Sigma), 1 mM Dithiothreitol (DTT) (Sigma), 0.5 mM PMSF (Sigma), 5 µg/ml Aprotinin (Sigma)]. Cell suspensions were incubated on ice for 15 minutes, then 25 µl of 10% (v/v) Nonidet P-40 (Sigma) were added and the tubes were vigorously vortexed for 10 seconds. To separate cytoplasmic proteins from crude intact nuclear, the homogenates were centrifuged for 30 seconds at 12,000 rpm at 4°C. After removal of the supernatant containing cytoplasm

proteins, crude intact nuclei pellets were resuspended in 100 µl of the ice cold/high ionic strength lysis buffer C (20 mM Hepes pH 7.9, 400 mM NaCl, 1 mM EDTA pH 8, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 5 µg/ml Aprotinin) which is able to disrupt the nuclear membrane and to selectively extract nuclear proteins. The tubes were vigorously rocked at 4°C for 15 minutes on a shaking platform (RotoMix; P.B.I. International), then the homogenates were centrifuged 5 minutes at 12,000 rpm at 4°C and supernatants containing the nuclear proteins were transferred in a clean 1.5 ml conical tube.

Protein concentration was determined as described in section 3.1.4, and nuclear extracts were stored at -80°C as 50 µg aliquots.

3.2.3 Western blot analysis

Equivalent amounts of nuclear extracts were fractionated, blotted onto solid support and immunoassayed to qualitatively evaluate the expression of MMR proteins (hMLH1, hPMS2 and hMSH2) and NER protein ERCC1 in OAW42, OAW42Mer, A2780, A2780cp8 and A2780d cell lines. Nuclear extracts from HeLa ($hMLH1^+$, $hPMS2^+$, and $hMSH2^+$), LoVo ($hMLH1^+$, $hPMS2^+$, and $hMSH2^-$) and HCT-116 ($hMLH1^-$, $hPMS2^-$, and $hMSH2^+$) cells were used as controls for MMR proteins basal expression.

3.2.3.1 SDS-polyacrylamide gel electrophoresis

An 8% (w/v) polyacrylamide resolving gel (linear range separation = 36-94 KDa) was used to obtain the separation of hMLH1, hMSH2 and hPMS2 proteins (86, 100 and 110 KDa respectively). An aliquot of OAW42, OAW42Mer, A2780, A2780d and A2780cp8 nuclear extracts containing 100 μg of proteins were mixed with 20 μl of 'Colour markers for SDS-PAGE and Protein transfer' spanning a molecular weight range of 6.5-205 KDa (Sigma) in a 1.5 conical tube. An equal volume of 2x SDS loading buffer (100 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue (Sigma), 20 (v/v) glycerol and 200 mM DTT) was then added. Tubes were heated at 100°C for 5 minutes (Modular-Thermostat; P.B.I. International), chilled on ice for an additional 5 minutes, centrifuged at 4°C at 12,000 rpm for 5 seconds and loaded onto the SDS-PAGE system.

Proteins were fractionated running the gel at constant voltage (55 volt over night) or 8 volt/centimetres for stacking gel and 15 volt/centimetres when the dye front was moved into the resolving gel at room temperature by means of a Biorad 200/2.0 power supply (Biorad). The run was stopped when the 66 KDa band of the colour marker reached the bottom of the gel.

The same procedure, with the exception of the resolving gel percentage (12% w/v) corresponding to a 14-60 KDa linear range of separation, was used to separate ERCC1 (36 KDa) protein. In this case, the run was stopped when the 45 KDa band of the colour marker reached the bottom of the gel.

3.2.3.2 Transfer of fractionated proteins from SDS-polyacrylamide gel to nitrocellulose filter

Protein blotting was performed in a 'Trans-Blot Cell' electrophoresis protein transfer unit (Biorad). When the SDS-PAGE was approaching the end of its run, four pieces of Whatman 3MM (Whatman International LtD., Maidstone, UK) and one piece of nitrocellulose filter (Hybond ECL, Amersham-Pharmacia Biotech.) to the exact size of the resolving gel were cut and soaked for at least 5 minutes in transfer buffer [50 mM Tris base, 100 mM Glycine, 0.01% (w/v) SDS, 20% (v/v) methanol (Carlo Erba Reagenti), pH 7.5]. At the end of the run, the glass plates holding the SDS-polyacrylamide gel were removed from the electrophoresis tank, the glass plates were opened and, by means of a scalpel, the stacking gel was removed. The resolving gel was placed exactly on the top of nitrocellulose filter and then sandwiched between two pairs of transfer buffer wetted-Whatman 3MM papers. Possible air bubbles trapped were displaced by repeated rolling with a pipette tip. The sandwich was then placed between two transfer buffer wetted-porous pads and two plastic supports and the entire construction was immersed in the electrophoresis transfer tank containing transfer buffer with the nitrocellulose filter placed toward the anode.

Transfer of the protein from the gel to nitrocellulose membrane was carried out at room temperature for 2 hours at 60 volt (constant voltage) by means of a Biorad 200/2.0 power supply.

3.2.3.3 Immunological detection of nitrocellulose-immobilised proteins

At the end of the transfer, the nitrocellulose filter was recovered from the sandwich and, in order to verify the correct protein blotting, it was stained for 5 minutes in a clean tray containing a "Ponceau S red dye" solution (Sigma) with gentle agitation at room temperature. When the bands of proteins were visible, the filter was destained with several washes of distilled water at room temperature.

On the basis of the molecular weight marker position, membrane slices containing proteins corresponding to about 36 KDa (ERCC1), 57 KDa (tubulin β), 86 KDa (hMLH1), 100 KDa (hMSH2) and 110 KDa (hPMS2) were excised with a scalpel and placed in separate clean trays. To completely destain the Ponceau S red dye bound to the proteins, nitrocellulose slices were washed three times for 20 minutes at room temperature on a shaking platform with blocking solution [5% (w/v) dried-nonfat milk (Regilait-Barcelona, Spain), 0.1% (v/v) Tween-20 (Sigma) and PBS], filter slices were then incubated at room temperature on a shaking platform in blocking solution to mask potential nonspecific antibodies binding sites and, thus, to reduce general background. After 1 hour, blocking solution was removed and filter slices were incubated over night at 4°C on a shaking platform with the specific antibody-containing solution. Detection of proteins of interest was achieved by using polyclonal antibodies anti hMLH1, hMSH2 and hPMS2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or monoclonal antibodies anti ERCC1 and tubulin β (NeoMarkers, Fremont, CA, USA), diluted to a final concentration of 1 μg/ml

in blocking solution. At the end of the over night incubation, primary antibodies were removed and filter slices were washed 3 times for 20 minutes with T-PBS wash solution [0.1% (v/v) Tween-20 in PBS] at room temperature on a shaking platform. Nitrocellulose filters were then incubated at room temperature on a shaking platform with the secondary antimouse or antirabbit Ig horseradish peroxidase-linked whole antibodies (Amersham Pharmacia Biotech.) diluted 1:1000 in blocking solution. After a 1-hour hybridisation, secondary antibodies were removed and filter slices were washed three times for 20 minutes at room temperature on a shaking platform with T-PBS wash solution followed by a rapid wash with PBS. Antibodies bound to proteins of interest on nitrocellulose slices were detected by means of the enhanced chemoluminescence system (ECL) (Amersham-Pharmacia Biotech.) according to the manufacturer's instructions using Hyperfilm MP films (Amersham-Pharmacia Biotech.).

3.2.4 Evaluation of ERCC1 gene expression by RT-PCR analysis

Basal levels of expression of ERCC1 gene in the OAW42, OAW42Mer, A2780, A2780d and A2780cp8 cells were quantified by RT-PCR analysis.

3.2.4.1 Cell preparation

Cells in logarithmic growth phase were detached from the flasks by means of sterile Trypsin-EDTA solution, transferred into a sterile a 15 ml conical tube

and pelleted by a 5-minute centrifugation at 1,500 rpm at 4°C. Single-cell suspension was obtained by resuspending cell pellets in 10 ml of sterile PBS, the suspension was checked under the microscope, and a small aliquot (100-500 µl) was transferred in the isoton solution and counted in the particle counter.

An aliquot of cell suspensions containing about 5-10 x 10^6 cells was then transferred in a sterile 1.5 ml tube, cells were pelleted by a 15-second centrifugation at 12,000 rpm at 4°C. After removal of PBS, cell pellets were washed twice with 1 ml of sterile PBS. At the end of the last wash, PBS was removed and total RNA was extracted from the cells.

3.2.4.2 Total RNA extraction

All extraction and manipulation steps were performed under sterile conditions using Diethylpirocarbonate (DEPC) (Sigma)-treated or 'RNase-free' certified chemicals and plastic disposable materials.

Total cellular RNA was obtained from fresh or frozen cell pellets by means of the 'Trizol Reagent' (Life Technologies Italia) according to the manufacturer's instructions. The amount of extracted RNA was determined spectrophotometrically.

Quality of isolated RNA was checked by gel electrophoresis analysis carried out by using a 1% (w/v) agarose mini gel (10 centimetres length/6 centimetres wide) prepared in 1x TAE buffer. An aliquot corresponding to about 1 µg of extracted ribonucleic acid was mixed in a RNase-free 0.5 ml tube with an equal

volume of sequencing gel loading buffer [95% (v/v) Formamide (Sigma), 20 mM EDTA pH 8, 0.05% (w/v) bromophenol blue and 0.05% (w/v) xylene cyanol (Sigma)], heated at 100°C for 3 minutes (in order to completely destroy possible RNA secondary structures), chilled on ice for further 3 minutes and loaded. RNA samples were run at a constant voltage of 90 volts for 1 hour at room temperature. The gel was then stained for 30 minutes at room temperature with gentle shaking in the ethidium bromide staining solution (1μg/ml ethidium bromide prepared in 1x TAE buffer) and destained for further 30 minutes at room temperature with gentle shaking in 1x TAE buffer. RNA was visualised by means of the UV transilluminator and photographed on a 667 Polaroid film (Polaroid LtD-St. Albans, UK). The presence of well defined and visible bands corresponding to both 28S and 18S ribosomal RNA was indicative of a good RNA preparation.

Total RNA extracted from each of the cell lines was stored at –80°C as 1μg aliquots.

3.2.4.3 Synthesis of first strand cDNA by reverse transcription reaction

Total RNA extracted from each cell line was reverse transcribed by using 'RNA PCR Core Kit' (Perkin-Elmer Italia). In a RNase-free 0.5 ml reaction tube (Perkin-Elmer Italia), 1 μg of total RNA was mixed with 2.5 μM random hexamers, 20 units of RNase inhibitor, 2.5 mM $MgCl_2$, 1mM each dNTP, 1x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 50 units of MuLV reverse transcriptase and RNase-free water to a final volume of 20 μl. Tubes were

incubated at room temperature for 15 minutes (to allow annealing between random hexamers and RNA). First strand cDNA synthesis and MuLV reverse transcriptase inactivation were then obtained by a 42°C-incubation for 30 minutes and a 5-minute heating at 95°C, respectively, in a hot-bonnet thermal cycler (MJ Research/M-Medical-Florence, Italy). Samples were stored at 4°C until they were used in PCR reactions.

3.2.4.4 PCR reactions

Primers used were synthesised and supplied in lyophilised form by M-Medical:

- ERCC1 sense (5'-GTGCAGTCGGCCAGGATACAC-3'; Tm = 62°C) corresponding to position 233-252 of human ERCC1 mRNA;
- ERCC1 antisense (5'-GTCCTCCTGGAGTGGCCAAG-3'; Tm = 65°C) corresponding to position 706-726 of human ERCC1 mRNA;

- β-Actin sense (5'-GGGAATTCAAAACTGGAACGGTGAAGG-3'; Tm = 60°C) corresponding to position 1336-1362 of human β-Actin mRNA;
- β-Actin antisense (5'-GGAAGCTTATCAAAGTCCTCGGCCACA-3'; Tm = 61°C) corresponding to position 1405-1431 of human β-Actin mRNA;

For each primer, 100 μM stock solution was prepared by dissolving the lyophilised primer in an opportune volume of 1x TE pH 8 (10 mM Tris-HCl, 1 mM EDTA) by extensive vortexing. Oligonucleotide concentration was

checked spectrophotometrically as described in section 3.1.3. The successive dilutions were prepared in distilled water.

Non-competitive quantitative PCR amplification was performed using the 'RNA PCR Core Kit' (Perkin-Elmer Italia) as follows. In DNase-free 0.5 ml PCR reaction tubes, 125 ng of total RNA reverse transcribed in first strand cDNA were mixed with 1 μM sense and antisense ERCC1 primers, 0.1 μM sense and antisense β-Actin primers, 0.625 units of AmpliTaq DNA polymerase, 1x PCR buffer, 2 mM $MgCl_2$ and DNase-free water to a final volume of 25 μl. Tubes were then placed in a hot-bonnet thermal cycler and both 494 bp ERCC1 and 96 bp β-Actin PCR products were obtained performing 1 cycle at 95°C for 5 minutes followed by 30 cycles each formed by a denaturation step of 1 minute at 95°C, an annealing step of 30 seconds at 65°C [Ta = (Tm sense primers + Tm antisense primers/4) + 3°C] and an extension step of 30 seconds at 72°C followed by a final extension cycle of 5 minutes at 72°C.

It is important to stress that these experimental conditions were selected, on the basis of preliminary experiments performed, to ensure that the PCR was still in the exponential phase when the reaction was stopped.

3.2.4.4.1 Gel electrophoresis and densitometric quantification of PCR products

Twenty-five μl of PCR solution were mixed with 5 μl of 6x gel loading buffer (0.25% (w/v) of both bromophenol blue and xylene cyanol, 30% (v/v)

glycerol in distilled water) and samples were loaded onto 3% (w/v)-1x TAE buffer agarose gel. Electrophoretic separation was performed at 90 volts in 1x TAE buffer and was stopped when bromophenol blue dye reached the bottom of the gel. Agarose gel was then stained for 30 minutes at room temperature with gentle shaking in the ethidium bromide-1x TAE buffer staining solution and destained for a further 30 minutes at room temperature with gentle shaking in 1x TAE buffer. PCR products were visualised on the UV-transilluminator and photographed on a 665 Polaroid film. Negative copy of the film was developed by using 18% (w/v) sodium sulphite (Sigma) according to the manufacturer's instructions and it was used for densitometric quantification of the PCR products by means of a HP ScanJet IIcx/t scanner (HewlettPackard Italia-Milan, Italy) and IQ software (Molecular Dynamics Ltd., Kemsing, UK).

3.2.5 Evaluation of the "alternatively-spliced form/full-length form" ratio of ERCC1 mRNA by RT-PCR analysis

The ratio between alternatively spliced form lacking exon VIII (ΔexVIII) and full-length form of the ERCC1 mRNA in the OAW42, OAW42Mer, A2780, A2780d and A2780cp8 cells was evaluated by RT-PCR analysis.

3.2.5.1 PCR reactions

Primers used were synthesised and supplied in lyophilised form by M-Medical:

- ERCC1 sense (5'-GAGCTGGCTAAGATGTGTATCC-3'; Tm = 60°C) corresponding to position 692-713 of human ERCC1 mRNA;
- ERCC1 antisense (5'-AGGCCAGATCTTCTCTTGATGC-3'; Tm = 60°C) corresponding to position 938-959 of human ERCC1 mRNA;
- β-Actin sense (5'-ACACTGTGCCCATCTACGAGG-3'; Tm = 60°C) corresponding to position 555-575 of human β-Actin mRNA;
- β-Actin antisense (5'-AGGGGCCGGACTGCTCATACT-3'; Tm = 62°C) corresponding to position 1155-1175 of human β-Actin mRNA;

For each primer, 100 μM stock solution was prepared by dissolving the lyophilised primer in an opportune volume of 1x TE pH 8 by extensive vortexing. Oligonucleotide concentration was checked spectrophotometrically as described in section 3.1.3. The successive dilutions were prepared in distilled water.

First strand cDNA previously synthesised (3.2.4.3 section) was used as template in non-competitive quantitative PCR amplification. PCR reactions were carried out as described in the 3.2.4.4 section with the exception that an annealing temperature of 60°C [(Ta = Tm sense primers + Tm antisense primers/4)] instead of 65°C [Ta = (Tm sense primers + Tm antisense primers/4) + 3°C] was used.

3.2.5.2 Gel electrophoresis and densitometric quantification of PCR products

ERCC1 full-length form (267 bp), ERCC1 alternatively spliced (ΔexVIII) form (196 bp) and β-actin (621 bp) PCR products were separated, visualised and quantified exactly as described in the 2.2.4.4.1 section with the exception that a 1.5% (w/v) agarose gel instead of a 3% (w/v) agarose gel was used.

3.2.6 Microsatellite instability (MIN) analysis

MIN analysis was carried out to evaluate the MMR functional status in OAW42, OAW42Mer, A2780, A2780d and A2780cp8 cell lines.

3.2.6.1 Genomic DNA extraction

Genomic DNA was extracted from the five cell lines as described by Sunters (Sunters et al., 1996) with minor modifications.

OAW42, OAW42Mer, A2780, A2780d and A2780cp8 cells in logarithmic growth phase were detached from the flasks with Trypsin-EDTA solution, transferred into a 15 ml conical tube and pelleted by a 5-minute centrifugation at 1,500 rpm at 4°C. Single-cell suspension was obtained by resuspending cell pellets in 10 ml of PBS, checked under the microscope, and a small aliquot (100-500 μl) was transferred in the isoton solution and counted in the particle counter. An aliquot of cell suspensions corresponding to about 1 x 10^6 cells

was then transferred in a 1.5 ml tube and cells were pelleted by a 15-second centrifugation at 12,000 rpm at 4°C. After removal of PBS, cell pellets were washed twice with 1 ml of sterile PBS and, at the end of the last wash, PBS was removed. Cell pellets were gently resuspended with 340 µl of lysis buffer [400 mM Tris-HCl pH 8, 60 mM EDTA pH 8, 150 mM NaCl, 1% (w/v) SDS, 10 µg/ml RNase A (Sigma)] and incubated at 37°C for 30 minutes. One hundred µl of 5 M sodium perchlorate (Sigma) were then added to each sample and tubes were firstly mixed in a shaking platform for 20 minutes at room temperature before being incubated for 20 minutes at 65°C with occasional agitation. Samples were cooled at room temperature, 580 µl of -20°C-cold chloroform (Carlo Erba Reagenti) were added and tubes were mixed in a shaking platform at room temperature. After 20 minutes, samples were centrifuged at 12,000 rpm for 10 minutes at 4°C and the upper phase containing DNA was transferred in a clean 1.5 ml tube. To precipitate genomic DNA, 2 volumes of -20°C-cold absolute ethanol were added to each tube. Cellular DNA was pelleted by a 12,000 rpm centrifugation at 4°C for 3 minutes and DNA pellets were washed twice with -20°C-cold 70% (v/v) ethanol, air dried and dissolved in an opportune volume of DNase-free water by an over night incubation at 4°C on a shaking platform.

DNA concentration was determined spectrophotometrically. DNA quality was checked by electrophoretic analysis on a 1% (w/v) agarose mini gel (prepared and run in 1x TAE buffer) in which about 1 µg of each DNA sample was separated at 90 volts at room temperature for 1 hour and visualised by UV-

transilluminator after a 30 minutes-ethidium bromide-1x TAE buffer staining and 30 minutes 1x TAE buffer destaining.

3.2.6.2 Primers

Oligonucleotides used for MIN analysis were synthesised and supplied in lyophilised form by M-Medical:

Locus D2S123:

Sense: 5'-ACAGGATGCCTGCCTTTA-3' (Tm = 53°C);

Antisense; 5'-ACTTTCCACCTATGGGAC-3' (Tm = 57°C);

Locus D17S250:

Sense: 5'-AGAATCAAATAGACAAT-3' (Tm = 47°C);

Antisense: 5'-GCTGGCCATATATATATTTAAACC-3' (Tm = 59°C);

Locus BAT26:

Sense: 5'-GACTACTTTTGACTTCAGCC-3' (Tm = 55°C);

Antisense: 5'-CCATTCAACATTTTTAACCC-3' (Tm = 53°C);

Locus BAT40:

Sense: 5'-TAACTTCCTACACCACAAC-3' (Tm = 53°C);

Antisense: 5'-AGAGCAAGACCACCTT-3' (Tm = 53°C);

Locus D6S261:

Sense: 5'-GTGAAACCCTGTCTCACTGC-3' (Tm = 61°C);

Antisense: 5'-GGATTTATAGGACCATGCCA-3' (Tm = 63°C);

Locus D3S1283:

Sense: 5'-GGCAGTACCACCTGTAGAAATG-3' (Tm = 57°C);

Antisense: 5'-GAGTAACAGAGGCATCGTGTATTC-3' (Tm = 53°C);

Locus EDH17B:

Sense: 5'-GTGACCCACGAAACACAGG-3' (Tm = 59°C);

Antisense: 5'-CAGAAGGTGAAGAACTCATCCA-3' (Tm = 58°C);

Locus GATA11B12:

Sense: 5'-AACAAAACAAAACAAAACAAACA-3' (Tm = 50°C);

Antisense: 5'-GGTGGAAATGCCTCATGTAG-3' (Tm = 57°C);

Locus GGAA2E02:

Sense: 5'-AGGAAAGAGAAAGAAAGGAAGG-3' (Tm = 56°C);

Antisense: 5'-TATATGATGAAAGTATATTGGGGG-3' (Tm = 56°C);

Locus D6S1035:

Sense: 5'-ACTTGAATCCAGGCATTCAG-3' (Tm = 55°C);

Antisense: 5'-AAAACTCAAGCTCAGAAAGGC-3' (Tm = 56°C);

For each primer, stock solution and successive dilutions were prepared as described in the 3.2.4.4 section.

3.2.6.3 PCR reactions

In clean 0.5 ml PCR reaction tubes, 20 ng of genomic DNA isolated from each of the five cell lines under investigation were mixed with 1 μM of both sense and antisense primers, 3 μCi of [α-^{32}P]-dCTP (3,000 Ci/mmole) (Amersham-Pharmacia Biotech Italia), 200 μM of each dNTP (Takara Biomedicals, Japan), 1x Ex-Taq PCR buffer (2 mM $MgCl_2$, 10 mM Tris-HCl pH 8.3, 50 mM KCl) (Takara Biomedicals) and distilled water to a final

volume of 5 µl. Tubes were placed in the hot-bonnet thermal cycler and heated at 95°C for 5 minutes. Five µl of an aqueous solution containing 0.5 units of Ex-Taq DNA polymerase (Takara Biomedicals) was then added to each tube and samples were subjected to the amplification reaction for 30 cycles each composed of a denaturation step of 30 seconds at 94°C, an annealing step of 30 seconds at a temperature, equivalent to the mean melting temperature of the sense and antisense primers plus 5°C [(Tm primer sense + Tm primer antisense/2) + 5°C], and an extension step of 30 seconds at 72°C, followed by one extension cycle of 7 minutes at 72°C.

At the end of the amplifications, 10 µl of sequencing gel loading buffer were added to each tube and samples were stored at –20°C when not immediately subjected to electrophoretic separation.

3.2.6.4 Sequencing gel electrophoresis

Sequencing gel was prepared as described in the 3.1.7 section.

PCR samples were heated at 95°C for 5 minutes, chilled on ice for a further 5 minutes and then quickly centrifuged at 4°C. Sequencing gel pre-run was stopped and each well was washed with 1x TBE to remove any trace of urea. Four µl of each heat-denatured PCR sample were then loaded. Electrophoretic separation was performed at a constant power of 57 watt and was stopped when bromophenol blue dye reached the bottom of the gel.

At the end of the electrophoresis run, the gel mold was removed from the apparatus and, after that the polyacrylamide gel was cooled to room

temperature, glass plates were carefully separated. Sequencing gel was detached from the non silicone-treated glass plate by means of Whatman 3MM papers and, after that a sheet of Saran Wrap was placed on the top, the gel was dried at 80°C in a 583 Gel Dryer (Biorad). After 40 minutes, the sequencing gel was removed from the dryer and placed in a Hypercassette (Amersham-Pharmacia Biotech.) with intensifying screens and autoradiographed by exposure to x-ray film (Hyperfilm; Amersham-Pharmacia Biotech.) over night at –80°C.

The presence of instability at different loci was independently evaluated by three different observers.

3.2.7 Intracellular drug accumulation

Each experimental sample was run in triplicate. Cellular platinum levels were expressed as micrograms of platinum per cell number where the cell number being determined by counting parallel cultures. The experiments were repeated at least three times.

3.2.7.1 Sample preparation

After harvesting in logarithmic growth phase, 2 x 10^6 cells were seeded in 90-millimetres dishes (Corning-Costar Italia) containing 10 ml of fresh medium. Dishes were incubated at 37°C in a 5% CO_2 humidified atmosphere in air incubator for 24 hours. As regards the OAW42Mer cell line, it is

important to stress that melphalan was not added to the growth medium for at least 2 passages before initiation of the experiment.

Immediately before cell treatment, cisplatin (3.3 mM) and BBR 3464 (807.1 μM) stock solution were diluted in specific growth media to obtain the different 11x working solutions of the two drugs. One ml of each 11x drug working solution were added to each dish (containing attached cells and 10 ml of medium) in order to expose the cells to 1x final drug concentration in a final volume of 11 ml. For untreated control samples, 1 ml of fresh medium was added. After a 1-hour incubation of cultures at 37°C in a 5% CO_2 humidified atmosphere in air incubator, drug-containing medium was removed from each dish, then cell monolayers were washed three times by immersing 90-mm dishes in ice-cold PBS, air dried and harvested at room temperature.

3.2.7.2 Atomic absorption spectrometry

Analysis for platinum content was performed by atomic absorption spectrometry (model 3;300) (Perkin-Elmer Italia) in the laboratories of the Department of Medical Sciences, Università degli Studi di Torino-Novara, Italia.

3.2.8 Platinum bound to genomic DNA

Each experimental sample was run in triplicate. The levels of platinum bound to cellular DNA were expressed as picograms of platinum per micrograms of DNA. The experiments were repeated at least three times.

3.2.8.1 Sample preparation

After harvesting in logarithmic growth phase, 10^7 cells of OAW42, OZW42Mer, A2780, A2780d and A2780cp8 cell lines were seeded onto 75 cm^2 culture flasks (Corning-Costar, Milano) in 15 ml of fresh medium and placed in the incubator for 24 hours. As regards the OAW42Mer cell line, it is important to stress that melphalan was not added to the growth medium for at least 2 passages before starting the experiment.

Immediately before cell treatment, cisplatin (3.3 mM) and BBR 3464 (807.1 μM) stock solution were diluted in specific growth media to obtain the different 11x working solutions of the two drugs. One point five ml of each 11x drug working solution were added to each flask (containing attached cells and 15 ml of medium) in order to expose the cells to 1x final drug concentration in a final volume of 16.5 ml. For untreated control samples, 1.5 ml of fresh medium was added. After a 1-hour incubation of cultures at 37°C in a 5% CO_2 humidified atmosphere in air incubator, drug-containing medium was removed from each flasks and cell monolayers were washed twice with 20 ml of PBS and, subsequently, detached with 2 ml of Trypsin-EDTA solution at

37°C for 5 minutes. In each flask, trypsinisation was blocked by addition of 20 ml of 10% (v/v) FBS-PBS solution, then cell suspension were centrifuged in a 50 conical tube at 1,500 rpm for 5 minutes at 4°C and washed twice with 30 ml of PBS. Cells pellets were then resuspended in 1 ml of PBS and transferred in a 1.5 ml tube. Single-cell suspension was checked under the microscope and a small aliquot (100-500 µl) was transferred in the isoton solution and counted in the particle counter.

Single cell suspension was pelleted by a 30-second centrifugation at 12,000 rpm at 4°C and, after PBS removal, genomic DNA was isolated from the cells, quantified and checked as described in the 3.2.6.1 section.

3.2.8.2 Coupled Plasma Mass Spectroscopy

Analysis of platinum bound to genomic DNA of each cell sample was performed by means of coupled plasma mass spectroscopy as described by Bonetti et al. (1996). The analysis was carried out in the laboratories of the Department of Pharmacology, Università degli Studi di Verona-Verona, Italia.

3.2.9 Evaluation of BBR 3464 and cisplatin ability to produce interstrand cross-links *in vitro*

BBR 3464 and cisplatin ability to induce interstrand cross-links on a naked plasmid DNA was evaluated according to the method described by Hartley et

al. (1991) with some modifications using the pSP73 vector (2464 bp) as template.

3.2.9.1 Linearisation of the pSP73 vector template with the HindIII restriction enzyme

In a 1.5 ml tube, an aliquot containing 30 µg of pSP73 plasmid was mixed with 60 units of HindIII restriction enzyme (Promega Italia), 1x of digestion buffer specific for HindIII (Buffer E) (Promega Italia) and distilled water to a final volume of 100 µl. The mixture was then incubated at 37°C in a water bath. After a 1 hour, the reaction was stopped by addition of 5 µl of 0.5 M EDTA, pH 8. The linearisation of the vector template was verified as described in the 3.1.9.3 section by loading about 200 ng of digested vector with the same amount of undigested plasmid DNA and 500 ng of λ-BstEII molecular weight marker.

3.2.9.2 Purification of HindIII-digested pSP73 vector

Digestion mixture was diluted to a final volume of 400 µl with distilled water. An equal volume of phenol (Sigma)/chloroform/isoamilic alcohol (Carlo Erba Reagenti) solution [25:24:1, (v/v/v)] was then added. The tube was extensively mixed by tapping and centrifuged at 12,000 rpm at 4°C for 5 minutes, then the aqueous upper phase, containing plasmid DNA, was recovered and placed in a clean 1.5 ml tube and an equal volume of

chloroform/isoamilic alcohol solution [24:1, (v/v)] was added. After mixing, the tube was centrifuged and the aqueous upper phase was recovered and placed in a clean 1.5 ml conical tube. To precipitate plasmid DNA, the aqueous phase was mixed with sodium acetate, pH 5.2 (Sigma), (0.3 M final concentration) then, after addition of 2 volumes of –20°C-cold 100% ethanol, the solution was mixed for a few seconds using the vortex and placed at –80°C for 1 hour. The sample was then centrifuged at 12,000 rpm at 4°C for 30 minutes, and, after ethanol removal, pelleted DNA was washed twice with 70% (v/v) ethanol and dried for 3 minutes by means of a speed-vacuum centrifuge (Heraeus Centrivac). Dried pSP73 vector was then dissolved in distilled water and quantified spectrophotometrically.

3.2.9.3 Removal of 5' terminal phosphate groups

In a 1.5 ml tube, an aliquot containing 20 µg (12.5 pmoles) of HindIII-linearised vector was mixed with 20 units of calf intestinal alkaline phosphatase (CIAP) (Promega), 1x CIAP buffer (Promega) and distilled water to a final volume of 50 µl. Dephosphorylation reaction was performed by a 3-hour incubation at 37°C in a water bath. The reaction was blocked by addition of EDTA, pH 8, to a final concentration of 5 mM and a 1-hour incubation at 65°C. Dephosphorylated DNA was then purified and quantified as described in the 3.2.9.2 section.

3.2.9.4 Labelling of linearised vector at 5' termini

In a 0.5 ml tube, 10 μg (6.25 pmoles) of linearised-dephosphorylated pSP73 vector were mixed with 40 units of T4 polynucleotide kinase (Takara Biomedicals), 60 μ Ci [γ-^{32}P]-ATP (3,000 Ci/mmole) (Amersham-Pharmacia Biotech), 1x T4 polynucleotide kinase buffer (Takara Biomedicals) and distilled water to a final volume of 50 μl. Labelling reaction was performed by incubating the tube at 37°C for 1 hour. T4 polynucleotide kinase was then inactivated by incubating the mixture at 95°C for 5 minutes.

Labelled DNA was purified and recovered as described in the 3.2.9.2 section with some modifications. To completely remove untransferred ^{32}P groups, labelled DNA pellet was washed 4 times with 70% ethanol; the dried DNA pellet was dissolved in distilled water (50 μl) and cpm corresponding to 1 μl of this DNA solution were evaluated by "Cerenkov counting" at the β-counter (Tri-Carb Liquid Scintillation Analyzer; Camberra-Packard Italia-Milan, Italy). Assuming a DNA recovery of 50% after purification steps, a solution at a concentration of 100 ng/μl with a specific activity of 50,000-100,000 cpm/ng was generally obtained.

3.2.9.5 Treatment of labelled-pSP73 with cisplatin and BBR 3464

For each experimental point, 100 ng of labelled DNA were mixed in a 0.5 ml tube with appropriate concentrations of cisplatin, or BBR 3464, Tris-HCl pH 8

(10 mM final concentration) and distilled water to a final volume of 40 µl. Two untreated control samples were also prepared omitting the addition of the drugs. Treatment reactions were performed for 1 hour at 37°C in the dark and were stopped by addition of an equal volume (40 µl) of stop solution [0.6 M sodium acetate pH 5.2, 20 mM EDTA pH 8 and 100 µg/ml salmon sperm DNA (Sigma)]. Plasmid DNA from treated and untreated samples was precipitated as described in the 3.2.9.2. section.

3.2.9.6 Gel electrophoresis analysis

DNA pellets obtained from drug-treated samples and one untreated control sample (control D) were dissolved in 10 µl of 'strand separation buffer' (30% (v/v) DMSO (Sigma), 1 mM EDTA pH 8, 0.04% (w/v) bomophenol blue and 0.04% (w/v) xylene cyanol), the DNA pellet from the other untreated control sample (control UD) was dissolved in 10 µl of 'native gel loading buffer' (6% (w/v) saccarose (Sigma), 0.04% (w/v) bomophenol blue and 0.04% (w/v) xylene cyanol). All samples were subjected to Cerenkov counting to verify the amount of DNA recovered for each experimental point after ethanol precipitation.

Before loading, tubes corresponding to drug-exposed samples and control (D) sample were denatured for 5 minutes by incubation at 95°C and chilled on ice for an additional 5 minutes. The untreated control sample (UD) was directly loaded onto a 25 centimetres-wide/25 centimetres-length 1% (w/v) agarose gel prepared in 1x TAE buffer.

Electrophoretic separation was performed in 1x TAE as running buffer at a constant voltage of 30 volts at room temperature. After 16 hours, electrophoresis was stopped, gel was placed onto Whatmann 3MM papers and, after that a sheet of Saran Wrap was placed on the top, the gel was dried at 80°C in the gel drier. After 2 hours, the agarose gel was removed from the dryer and placed in a Hypercassette with intensifying screens and autoradiographed by exposure to X-ray film over night at –80°C.

Quantification of the bands was performed by densitometric scanning of the X-ray film.

3.2.10 Evaluation of the sequence specificity of BBR 3464 and cisplatin induced DNA covalent adducts: the Taq stop assay

The Taq stop assay was performed as described by Bubley et al. (1994), with some modifications, to determine the sequence preference of DNA lesions produced by the novel trinuclear platinum complex, with respect to cisplatin, by using *in vitro* drug-treated naked plasmid and genomic DNA as well as genomic DNA isolated after exposure of intact cells to the drugs as templates.

3.2.10.1 Taq stop assay on *in vitro* treated naked pSP73 plasmid DNA

pSP73 vector was obtained as described in the 3.1.10 section.

3.2.10.1.1 pSP73 vector linearisation, purification and quantification

In a 0.5 ml tube, a volume corresponding to 30 µg of pSP73 plasmid were mixed with 60 units of HaeII restriction enzyme (Promega Italia), 1x of digestion buffer specific for HaeII (Buffer B) (Promega Italia) and distilled water to a final volume of 100 µl. The mixture was then incubated at 37°C in a water bath. After a 1 hour, the reaction was stopped by addition of 5 µl of 0.5 M EDTA, pH 8, and the linearisation of the plasmid was verified as described in the 3.2.9.1 section. Purification of HaeII-linearised pSP73 vector was carried out as described in the 3.2.9.2 section. After spectrophotometric quantification, plasmid DNA concentration was adjusted with distilled water to a final concentration of 40 ng/25 fmole/µl.

3.2.10.1.2 *In vitro* drug treatment of HaeII-digested plasmid DNA

For each experimental sample, 4 µl of linearised pSP73 vector, corresponding to 100 fmoles (160 ng), were mixed in a 0.5 ml tube with opportunely selected concentrations of cisplatin or BBR 3464, 2.6 µl of 100 mM Tris-HCl pH 8 (final concentration = 10 mM) and distilled water to a final volume of 26 µl. Untreated control samples were prepared by adding PBS instead of the drugs. Damaging reactions were performed by incubating the tubes at 37°C in a water bath in the dark for 2 hours and then stopped by snap freezing in liquid nitrogen. Frozen samples were stored at –80°C until they were used in linear PCR reactions.

3.2.10.1.3 Labelling of Sp6 primer at 5' termini

Sp6 primer (5'-GATTTAGGTGACACTATAG-3'; Tm = 48°C) corresponding to position 2447-1 of the pSP73 vector consensus sequence (Fig. 21) was synthesised and supplied in the lyophilised form by M-Medical. One hundred μM stock solution and successive dilutions were prepared as described in the 3.2.4.4. section.

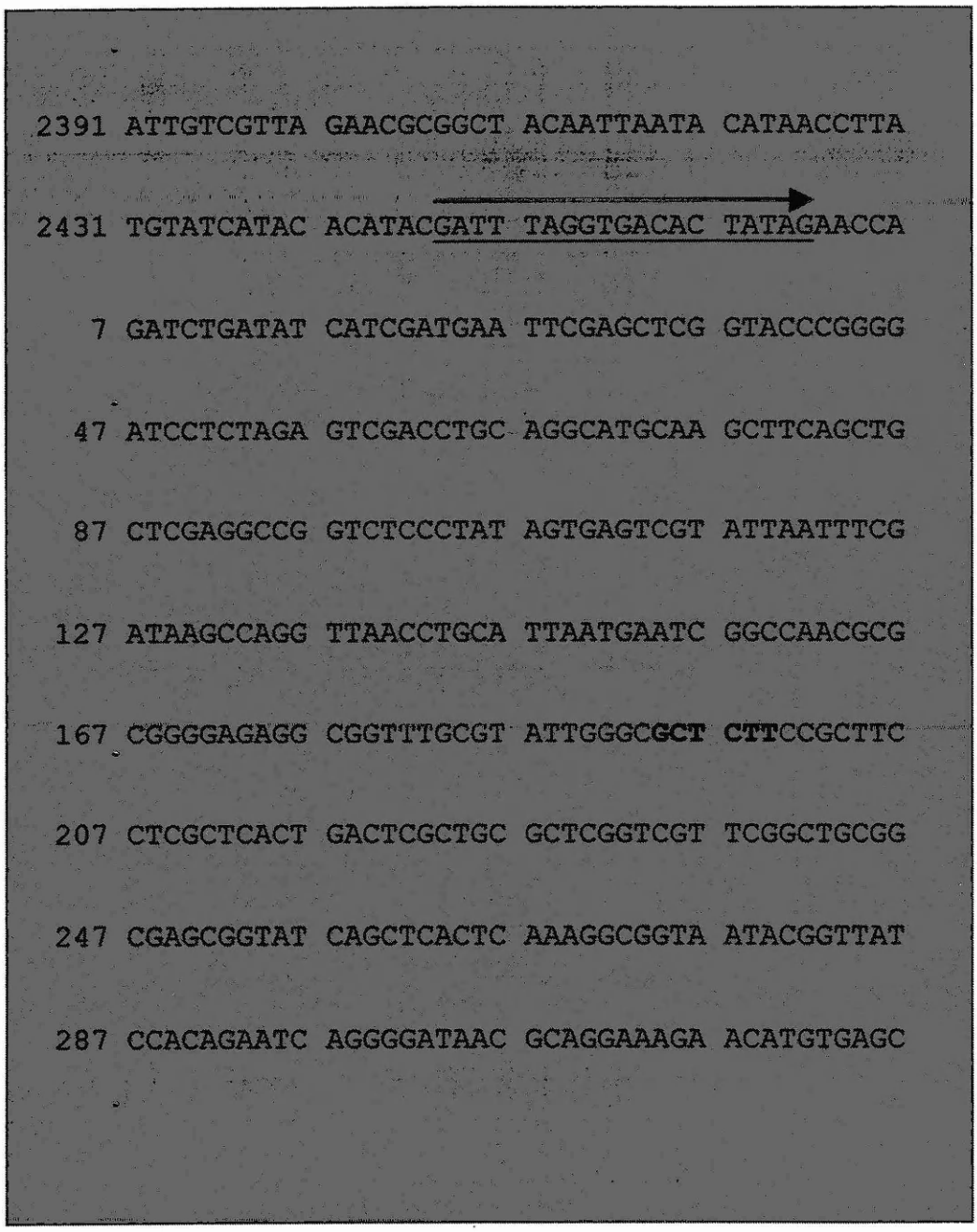
In a 0.5 ml tube, a volume corresponding to 300 pmole of Sp6 primer was mixed with

20 units of T4 polynucleotide kinase, 30 μ Ci [γ-^{32}P]-ATP (3,000 Ci/mmole), 1x T4 polynucleotide kinase buffer and distilled water to a final volume of 20 μl. Labelling reaction was performed by incubating the tube at 37°C for 1 hour. T4 polynucleotide kinase was then inactivated by incubating the mixture at 95°C for 5 minutes. The labelled primer was then stored at –20°C until it was used in linear PCR and/or in sequencing reactions.

3.2.10.1.4 Sequencing of the pSP73 vector

Sequencing of the pSP73 plasmid region included between Sp6 primer annealing region and HaeII-restricted site was performed by means of the AmpliCycle kit (Perkin-Elmer Italia) according to the method described by Lee et al. (Lee et al, 1991), that is a modification of the dideoxy chain termination procedure described by Sanger et al. (Sanger et al., 1977).

Figure 21. Sequence of the pSP73 plasmid DNA region subjected to determination of the sequence specificity of cisplatin- and BBR 3464-induced lesions by Taq stop assay. The underlined sequence indicates the position of the Sp6 'sense' primer (→). In bold are indicated the bases of the HaeII site.


2391 ATTGTCGTTA GAACGCGGCT ACAATTAATA CATAACCTTA
2431 TGTATCATAC ACATACGATT TAGGTGACAC TATAGAACCA
7 GATCTGATAT CATCGATGAA TTCGAGCTCG GTACCCGGGG
47 ATCCTCTAGA GTCGACCTGC AGGCATGCAA GCTTCAGCTG
87 CTCGAGGCCG GTCTCCCTAT AGTGAGTCGT ATTAATTTCG
127 ATAAGCCAGG TTAACCTGCA TTAATGAATC GGCCAACGCG
167 CGGGGAGAGG CGGTTTGCGT ATTGGGCGCT CTTCCGCTTC
207 CTCGCTCACT GACTCGCTGC GCTCGGTCGT TCGGCTGCGG
247 CGAGCGGTAT CAGCTCACTC AAAGGCGGTA ATACGGTTAT
287 CCACAGAATC AGGGGATAAC GCAGGAAAGA ACATGTGAGC

-Figure 21-

In a clean 0.5 ml tube, 30 µl of 'reaction mix' were prepared by mixing an aliquot of the DNA plasmid, corresponding to 30 fmole (49 ng), with 1.5 pmole of ^{32}P-labelled Sp6 primer, 3 µl of 10x Cycling mix (final concentration = 1x) and distilled water. Four 0.5 ml PCR reaction tubes were placed on ice and labelled with A, T, G and C letters, then, 2 µl of A-termination mix, T-termination mix, G-termination mix and C-termination mix were poured on the A, T, G, C-labelled tubes respectively. At the end, 6 µl of 'reaction mix' were transferred to each of the four labelled tubes. Sequencing reactions were carried out by placing the tubes in the hot-bonnet thermal cycler and performing a linear PCR amplification composed of 1 cycle at 95°C for 1 minute followed by 25 cycles, each consisting of a denaturation step of 1 minute at 95°C, an annealing step of 1 minute at 48°C (Ta = Tm) and an extension step of 1 minute at 72°C. At the end of the amplifications, 4 µl of sequencing gel loading buffer were added to each sequencing tube and samples were stored at –20°C if not immediately subjected to electrophoretic separation.

3.2.10.1.5 Linear PCR reactions (Taq stop reactions)

In order to inactivate the drugs, 13 µl of drug-treated and untreated samples (containing 50 fmole of pSP73 vector; section 3.2.10.1.2) were placed in a 0.5 ml PCR reaction tube, heated at 95°C for 5 minutes and then rapidly chilled on ice for further 5 minutes. After a brief 12,000 rpm-centrifugation at 4°C, 30

pmole (2 µl) of ^{32}P-labelled Sp6 primer, 1 unit of Ex-Taq DNA polymerase, 200 µM of each dNTP, 1x PCR Buffer, 5% (v/v) DMSO and distilled water, to a final volume of 20 µl were added to each 13 µl-DNA sample. Linear PCR reactions were carried out by placing the tubes in the hot-bonnet thermal cycler and performing 30 cycles, each consisting of a denaturation step of 20 seconds at 95°C, an annealing step of 1 minute at 48°C (Ta = Tm) and an extension step of 1 minute at 72°C. At the end of the amplifications, 6 µl of sequencing gel loading buffer were added to each tube and samples were stored at –20°C if not immediately loaded onto sequencing gel.

3.2.10.1.6 Sequencing gel electrophoresis

pSP73 sequencing and Taq stop reactions were loaded and separated in the same sequencing gel as described in the 3.1.7 and 3.2.6.4 sections.

3.2.10.2 Taq stop assay on *in vitro* treated naked genomic DNA

DNA was isolated from A2870 cells as described in the 3.2.6.1 section.

3.2.10.2.1 EcoRI genomic DNA digestion, purification and quantification

In a 1.5 ml tube, an aliquot corresponding to 60 µg of cellular DNA was mixed with 300 units of EcoRI restriction enzyme (Promega Italia), 1x of digestion buffer specific for EcoRI (Buffer H) (Promega Italia) and distilled

water to a final volume of 300 µl.The mixture was then incubated at 37°C in a water bath for 18 hours. The reaction was stopped by the addition of 5 µl of 0.5 M EDTA, pH 8. The DNA digestion was verified as described in 3.2.9.1 section by loading about 500 ng of both digested and undigested genomic DNA. Purification was carried out as described in the 3.2.9.2 section. After spectrophotometric quantification, DNA concentration was adjusted with distilled water to a final concentration of 750 ng/µl.

3.2.10.2.2 *In vitro* drug treatment of EcoRI-digested cellular DNA

For each experimental sample, 4 µl of digested genomic DNA corresponding to 3 µg were mixed in a 0.5 ml tube with opportunely selected concentrations of cisplatin or BBR 3464, 2.6 µl of 100 mM Tris-HCl pH 8 (final concentration = 10 mM) and distilled water to a final volume of 26 µl. Untreated control samples were prepared by adding PBS instead of the drugs. Damaging reactions were performed by incubating the tubes at 37°C in a water bath in the dark for 2 hours and then stopped by snap freezing in liquid nitrogen. Frozen samples were stored at –80°C until they were used in linear PCR reactions.

3.2.10.2.3 Labelling of the 16mer primer at 5' termini

16mer primer (5'-AATTCTCAGTAACTTC-3'; Tm = 41°C) corresponding to position 1-16 of the 340 bp repeat (also called α-RI DNA) consensus

sequence of the alphoid cellular DNA region (Fig. 18) was synthesised and supplied in the lyophilised form by M-Medical. One hundred μM stock solution and successive dilutions were prepared as described in the 3.2.4.4 section. The primer was labelled as described in the 3.2.10.1.3 section.

3.2.10.2.4 Sequencing of the α-RI DNA region

Sequencing of the α-RI DNA region upstream of the 16mer primer was performed as described in the 3.2.10.1.4 section with the following variants:

- ^{32}P-labelled 16mer primer instead of ^{32}P-labelled Sp6 primer;
- 300 ng of naked whole genomic DNA (corresponding to about 30 fmole of 340 bp α-RI DNA repeat) instead of 30 fmole (49 ng) of pSP73 vector;
- 51°C as annealing temperature (Ta = Tm + 10°C) instead of 48°C (Ta = Tm).

3.2.10.2.5 Linear PCR reactions (Taq stop reactions)

Taq stop reactions were performed by using 13 μl of drug-treated and untreated samples (containing 1.5 μg of genomic DNA). The procedure was identical to that described in the 3.2.10.1.5 section with the exception that 36 cycles instead of 30 cycles and an annealing temperature of 35°C (Ta = Tm – 6°C) instead of 48°C (Ta = Tm) were used.

3.2.10.2.6 Sequencing gel electrophoresis

Sequencing and Taq stop reactions were loaded and separated in the same sequencing gel as described in the 3.1.7 and 3.2.10.1.6 sections.

3.2.10.3 Taq stop assay on genomic DNA isolated after drug treatment of intact cells

3.2.10.3.1 Drug treatment of A2780 living cells

After harvesting in logarithmic growth phase, for each experimental point 10^6 A2780 cells were seeded in 6-well plates (Corning-Costar Italia) in 3 ml of fresh complete medium and incubated at 37°C in a 5% CO_2 humidified atmosphere in air incubator. After 24 hours, the complete medium was removed, cell monolayers were washed twice with 3 ml of PBS. Two ml of FBS-free medium were then added to each well.

Immediately before cell treatment, cisplatin (3.3 mM) and BBR 3464 (807.1 μM) stock solution were diluted in FBS-free specific growth medium to obtain the different 11x working solutions of the two drugs. Two hundred μl of each 11x drug working solution were added to each well (containing attached cells and 2 ml of medium) in order to expose the cells to 1x final drug concentration in a final volume of 2.2 ml. For untreated control samples, 200 μl of fresh FBS-free medium was added to each well. After a 2-hour incubation at 37°C in a 5% CO_2 humidified atmosphere in air incubator, drug-containing medium

was removed from each well, cells were washed three times with PBS (3 ml for each well) and detached from the plates by 5 minutes-incubation at 37°C with trypsin-EDTA solution. Trypsinisation was blocked by addition of 5 ml of 10% (v/v) FBS-PBS solution, then cell suspension was centrifuged in a 15 ml conical tube at 1,500 rpm for 5 minutes at 4°C and washed twice with 5 ml of PBS. Cells pellets were then resuspended in 1 ml of PBS and transferred in a 1.5 ml tube. Single-cell suspension was checked under the microscope and a small aliquot (100 µl) was transferred in the isoton solution and counted in the particle counter.

Single cell suspension was pelleted by 30-second centrifugation at 12,000 rpm at 4°C and, after PBS removal, genomic DNA was isolated from the cells, quantified and checked as described in 3.2.6.1 section.

3.2.10.3.2 EcoRI genomic DNA digestion, purification and quantification

Cellular DNA extracted from drug-treated and –untreated cells was digested with EcoRI restriction enzyme, purified, quantified and dissolved in distilled water to a final concentration of 750 ng/µl exactly as described in the 3.2.10.2.1 section.

3.2.10.3.3 Genomic DNA sequencing, Taq stop reactions and sequencing gel electrophoresis.

Sequencing and Taq stop reactions were performed, exactly as described in the 3.2.10.2.4 and 3.2.10.2.5 sections, on cellular DNA isolated from treated and untreated living cells.

Sequencing gel was prepared and run as described in the 3.2.10.2.6 section.

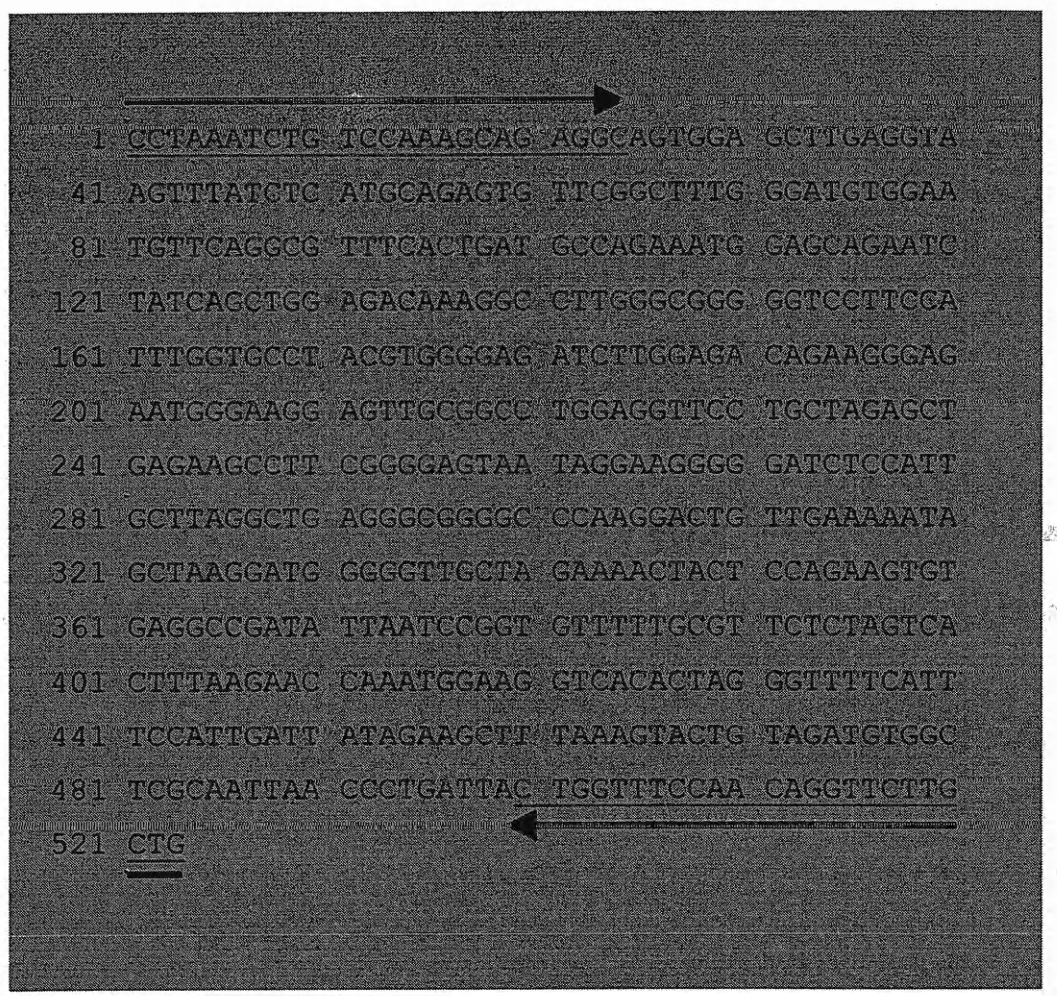
3.2.11 Assessment by quantitative PCR (QPCR) of gene-specific accumulation and repair of cisplatin- and BBR 3464-induced DNA damage

The formation and removal of drug-induced lesions in a 523 bp region including the intron 1 of the human N-*ras* gene was assessed by means of the QPCR assay essentially according to Bingham et al. (1996), with some modifications.

3.2.11.1 Primers

To select and amplify the 523 bp fragment a N-*ras* sense primer (5'-CCTAAATCTGTCCAAAGCAGAGGC-3'; Tm = 63°C) and a N-*ras* antisense primer (5'-CAGCAAGAACCTGTTGGAAACCAG-3'; Tm = 63°C) corresponding to positions 1-24 and 500-523 of the intron 1 of the human N-*ras* gene (Fig. 22), respectively, were used. These oligonucleotides were synthesised and supplied in the lyophilised form by M-Medical. Stock solution

Figure 22. Sequence of the Intron I of the human N-*ras* gene. The underlined sequences indicate the positions of the N-*ras* 'sense' (→) and 'antisense' (←) primers.


1 CCTAAATCTG TCCAAAGCAG AGGCAGTGGA GCTTGAGGTA
41 AGTTTATCTC ATGCAGAGTG TTCGGCTTTG GGATGTGGAA
81 TGTTCAGGCG TTTCACTGAT GCCAGAAATG GAGCAGAATC
121 TATCAGCTGG AGACAAAGGC CTTGGGCGGG GGTCCTTCGA
161 TTTGGTGCCT ACGTGGGGAG ATCTTGGAGA CAGAAGGGAG
201 AATGGGAAGG AGTTGCGGCC TGGAGGTTCC TGCTAGAGCT
241 GAGAAGCCTT CGGGGAGTAA TAGGAAGGGG GATCTCCATT
281 GCTTAGGCTG AGGGCGGGGC CCAAGGACTG TTGAAAAATA
321 GCTAAGGATG GGGGTTGCTA GAAAACTACT CCAGAAGTGT
361 GAGGCCGATA TTAATCCGGT GTTTTTGCGT TCTCTAGTCA
401 CTTTAAGAAC CAAATGGAAG GTCACACTAG GGTTTTCATT
441 TCCATTGATT ATAGAAGCTT TAAAGTACTG TAGATGTGGC
481 TCGCAATTAA CCCTGATTAC TGGTTTCCAA CAGGTTCTTG
521 CTG

-Figure 22-

and successive dilutions were prepared as described in the section 3.2.4.4 section.

3.2.11.2 Measurement of DNA lesions induced by cisplatin and BBR 3464 DNA *in vitro*

The formation of drug-induced platinum DNA lesions was assessed on naked whole genomic DNA exposed *in vitro* to cisplatin or BBR 3464.

3.2.11.2.1 Treatment of naked genomic DNA

For each experimental sample, 1 μg of naked genomic DNA, extracted from A2780 cells as described in the 3.2.6.1 section was mixed in a 0.5 ml tube with opportunely selected concentrations of cisplatin or BBR 3464, 10 μl of 100 mM Tris-HCl pH 8 (final concentration = 10 mM) and distilled water to a final volume of 100 μl. Untreated control samples were prepared by adding PBS instead of the drugs. Damaging reactions were performed by incubating the tubes at 37°C in a water bath in the dark for 1 hour and then stopped by snap freezing in liquid nitrogen. Frozen samples were stored at –80°C until they were used in QPCR reactions.

3.2.11.2.2 Quantitative PCR (QPCR)

To inactivate the drugs, 8 μl of drug-treated and untreated samples (containing 80 ng of whole naked genomic DNA; section 3.2.11.2.1) were

placed in a 0.5 ml PCR reaction tube, heated at 95°C for 5 minutes then rapidly chilled on ice for a further 5 minutes. For untreated control samples, an additional experimental point containing 4 µl (40 ng) of whole naked genomic DNA and 4 µl of distilled water was also set up. After a brief 12,000 rpm-centrifugation at 4°C, 0.5 µM of both N-*ras* sense and antisense primers, 0.6 units of Ex-Taq DNA polymerase, 120 µM of each dNTP, 1x of Ex-Taq PCR buffer, 0.3 µCi of [α-^{32}P]-dCTP (3,000 Ci/mmole) and distilled water to a final volume of 30 µl were added. Tubes were then placed in the hot-bonnet thermal cycler and subjected to the amplification reaction composed of 1 cycle at 95°C for 2 minutes followed by 26 cycles each consisting of a denaturation step of 1 minute at 95°C, an annealing step of 1 minute at 61°C [Ta = (Tm sense primer + Tm antisense primer/2) – 2°C] and an extension step of 1 minute at 72°C followed by a final extension cycle of 4 minutes at 72°C. It is important to stress that this thermal profile was selected on the basis of preliminary experiments, to ensure that the PCR was still in the exponential phase when the reaction was stopped. Samples were stored at 4°C until they were subjected to electrophoretic separation.

3.2.11.2.3 Polyacrylamide gel electrophoresis

To separate the 523 bp DNA band, a 5% (w/v) polyacrylamide-1x TBE buffered non-denaturing gel (resolution power = 80-500 bp) was prepared as described in the 2.1.8 section. Six µl of 6x gel loading buffer were then added

to each 30 µl-PCR tube and samples were loaded onto the gel. Electrophoretic run was performed at a constant voltage of 120 volts at room temperature. When the bromophenol blue dye reached the bottom of the gel (after about 4 hours from the start of the run), electrophoresis was stopped and the polyacrylamide gel was detached from the glass plates by means of Whatman 3MM papers. After that a sheet of Saran Wrap was placed on the top, the gel was dried at 80°C for 20 minutes, placed in a Hypercassette with intensifying screens and autoradiographed by exposure to X-ray film over night at –80°C.

3.2.11.2.4 Quantification of PCR products

The amount of 523 bp DNA fragments present in each experimental sample was quantified by densitometric analysis. The results were expressed as the integrated volume value of treated samples compared with that of untreated control samples.

3.2.11.3 Induction and removal of platinum DNA-adducts on living cells

The kinetics of induction and removal of platinum DNA-adducts was assessed on genomic DNA isolated from intact cells after treatment with cisplatin or BBR 3464.

3.2.11.3.1 Induction of DNA lesions in intact cells

After harvesting in logarithmic growth phase, 10^6 OAW42, OAW42Mer, A2780 or A2780cp8 cells, resuspended in 3 ml of growth media, were seeded in 60-millimetre dishes and incubated at 37°C in a 5% CO_2 humidified atmosphere in an air incubator. After 24 hours, the complete media was removed, cell monolayers were washed twice with 3 ml of PBS and then 2 ml of FBS-free media were then added to each dish.

Immediately before cell treatment, cisplatin (3.3 mM) and BBR 3464 (807.1 μM) stock solution were diluted in FBS-free specific growth medium to obtain the different 11x working solutions of the two drugs. Two hundred μl of each drug working solution were added to each well (containing attached cells and 2 ml of medium) in order to expose the cells to 1x final drug concentration in a final volume of 2.2 ml. For untreated control samples, 200 μl of fresh FBS-free medium was added to each well. After a 5-hour incubation at 37°C in a 5% CO_2 humidified atmosphere in air incubator, drug-containing medium was removed from each dish and cells were washed three times with PBS (3 ml for each dish). Cellular samples destined to be used in repair studies (T_6, T_{24} and T_{48}) were overlaid with 3 ml of complete media and incubated at 37°C for an additional 6, 24 or 48 hours, whereas cells destined for the measurement of platinum DNA lesions were collected immediately at the end of treatment (T_0).

3.2.11.3.2 Cell harvesting and genomic DNA extraction

At each time point, the growth medium was removed from the dishes and, after three washes with 3 ml of PBS, monolayers were harvested from the plates by means of a sterile cell scraper (Corning-Costar Italia), transferred to 1.5 ml tubes and pelleted by a 3,000 rpm centrifugation at 4°C for 10 minutes. Cell pellets were washed twice with 1.5 ml of PBS and resuspended in 1 ml of PBS. Single-cell suspension was checked under the microscope and cell number was determined for each sample by transferring a small aliquot (100 µl) in the isoton solution and counting it in the particle counter.

Single cell suspensions were then pelleted by a 30-second centrifugation at 12,000 rpm at 4°C and, after PBS removal, cellular pellets were resuspended in lysis buffer [50 mM KCl, 10 mM Tris-HCl pH8, 0.45% (v/v) Nonidet P-40, 0.45% (v/v) Tween-20, 60 µg/ml proteinase K (Sigma)], with a 'number of cells/volume of lysis buffer' ratio of 10^6 cells/160 µl, corresponding to 6,250 cell equivalents/µl.

Cellular proteins were degraded by incubating samples with proteinase K for 2 hours at 60°C. Tubes were then heated at 95°C for 15 minutes in order to completely denature and precipitate both proteinase K and degraded cellular proteins. Insoluble material containing cellular debris and denatured proteins was collected in the bottom of the tubes by a 12,000 rpm centrifugation at 4°C for 5 minutes. The DNA-containing aqueous phases were carefully removed and transferred into 0.5 ml tubes and diluted 1:100 in distilled water to achieve

a solution at a final concentration of 62.5 cell equivalents/µl. Samples were stored at 4°C until they were used in QPCR reactions.

3.2.11.3.3 Quantitative PCR (QPCR)

QPCR was performed as described in the 3.2.11.2.2 section with the following exceptions:

- 95°C-heating for 5 minutes of drug-treated and untreated samples to inactivate drugs was not performed;
- for drug-treated samples, an aliquot of 20 µl (corresponding to 1,250 cell/equivalents of genomic DNA) instead of an aliquot of 8 µl (corresponding to 80 ng of cellular DNA) was used;
- for untreated control samples, two aliquots of 20 µl and 10 µl (corresponding to 1,250 and 625 cell equivalents of genomic DNA respectively) instead of two aliquots of 8 µl and 4 µl (corresponding to 80 ng and 40 ng of cellular DNA respectively) were used.

3.2.11.3.4 Polyacrylamide gel electrophoresis and quantification of PCR products

Electrophoretic separation of PCR samples and quantification of 523 bp fragments were performed exactly as described in the 3.2.11.2.3 and 3.2.11.2.4 sections.

3.2.12 Treatment of OAW42 and OAW42Mer cell lines with cisplatin and BBR 3464 for the analysis of cell cycle disturbance and apoptosis

After harvesting in logarithmic growth phase, 2 x 10^6 OAW42 and OAW42Mer cells were seeded in 75-cm^2 flasks with 20 ml of fresh growth medium and were then incubated at 37°C in a 5% CO_2 humidified atmosphere in air incubator for 24 hours.

To expose cell lines to IC_{50} concentration of cisplatin (8.3 μM and 83 μM for OAW42 and OAW42Mer cell lines, respectively) and BBR 3464 (5.2 μM and 0.36 μM for OAW42 and OAW42Mer cell lines, respectively), 807.1 μM cisplatin (3.3 mM) and BBR 3464 (807.1 μM) stock solutions were diluted in growth medium to prepare different 11x working solutions (cisplatin = 91.3 μM and 913 μM for OAW42 and OAW42Mer cell lines respectively; BBR 3464 = 57.2 μM and 3.96 μM for OAW42 and OAW42Mer cell lines respectively) and 2 ml of the different 11x drug working solutions were added to each flask. For untreated control samples, 2 ml of fresh growth medium was added to each flask. After a 1-hour incubation at 37°C in a 5% CO_2 humidified atmosphere in air incubator, drug-containing medium was removed from each flask, cell monolayers were washed three times with 20-30 ml of PBS. Twenty ml of fresh growth medium was added to each flask and cultures were then incubated at 37°C in a 5% CO_2 humidified atmosphere in air incubator for additional 24 (T_{24} samples), 48 (T_{48} samples) or 72 (T_{72} samples) hours. At different intervals after drug treatment, the growth medium, containing floating

cells, was removed from the flasks and transferred in 50 ml conical tubes whereas attached cell monolayers were washed twice with 10 ml of PBS and detached by a 5-minute incubation at 37°C with trypsin-EDTA solution (2 ml for each flask). Trypsinisation was stopped by addition of a 10% (v/v) FBS-PBS solution (10 ml for each flask). Cellular suspensions were then pooled with the corresponding floating cells in 50 ml conical tubes and centrifuged at 1,500 rpm for 5 minutes at room temperature.

After 2 PBS washes, cell pellets were resuspended in 1 ml of PBS and transferred in 1.5 ml conical tubes. Single-cell suspensions were checked under the microscope and counted in a particle counter by mixing a small aliquot of cell suspension (generally 50 µl) with isoton solution.

Each experimental sample was split into various aliquots (containing an appropriate number of cells) for the different assays. Cells aliquots destined to be used in assays for which fresh cells were not required, were collected by a 12,000 rpm-centrifugation at 4°C for 30 seconds and frozen by snap freezing in liquid nitrogen and stored at –80°C.

3.2.12.1 Cell cycle analysis by flow cytometry

For T_{24}, T_{48}, and T_{72} (untreated control sample, cisplatin- and BBR 3464-treated samples) experimental points, 1 x 10^6 (0.5 ml) of fresh OAW42 and OAW42Mer cells were fixed by a drop-to drop addition of cellular suspension to 4.5 ml of –20°C cold-70% ethanol present in a 5 ml Nalgene tube. Fixed cells were stored at –20°C until they were subjected to the flow cytometric analysis. Immediately before analysis, fixed cells were collected by a 1,500

rpm-centrifugation at 4°C for 5 minutes, transferred into a 1.5 ml tube and washed twice with 0.5 ml of PBS. Cell pellets were then resuspended in 0.5 ml of the solution A [50 μg/ml propidium iodide (Sigma), 50 mg/ml RNase A and 0.05% (v/v) Nonidet P-40] and stained at 4°C. After 30 minutes, drug-induced cell cycle perturbations were assessed by analysing the stained cellular suspension with a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA, USA). At least 30,000 "events" were read and the percentages of cells in $G_{0/1}$, S and G_2/M phases were evaluated on DNA plots by CellFit software (Becton Dickinson) according to the SOBR model (Becton Dickinson).

3.2.13 Apoptosis induction studies

The possibility that activation of programmed cell death pathway occurred in the drug-treated OAW42 and OAW42Mer cell lines was investigated evaluating different endpoints.

3.2.13.1 Evaluation of the mitochondrial membrane potential ($\Delta\psi_{mt}$)

Since the disruption of mitochondrial membrane potential has been linked to the induction of apoptosis by different stimuli, possible changes of the $\Delta\psi_{mt}$ in the OAW42 and OAW42Mer cell lines treated with the two drugs were investigated.

These potential alterations were studied by means of a flow cytometry method (Salvioli et al., 1997) using the $\Delta\psi_{mt}$-sensitive dye JC-1 ($5.5^1 6.6^1$-tetrachloro-$1.1^1 3.3^1$-teraethylbenzimidazolyl carbocyanine iodide) (Molecular

Probes, Inc., Eugene, OR, USA). After cellular internalisation, the dye forms 590 nm wave length-emitting JC-1 aggregates at relatively high $\Delta\psi_{mt}$ whereas, in the absence of or at relatively low $\Delta\psi_{mt}$, internalised JC-1 exists as a 527 nm wave length-emitting monomeric form.

Forty eight hours after exposure to cisplatin or BBR 3464, (T_{48}), untreated and drug-treated OAW42 and OAW42Mer cells (0.5 x 10^6 cells) were collected by a 1,500 rpm-centrifugation for 5 minutes at room temperature. A positive control for $\Delta\psi_{mt}$ disruption was prepared by treating cells with valinomycin, a K^+ ionophore that uncouples oxidative phosphorylation. Zero point five x 10^6 cells of OAW42 and OAW42Mer cells were collected by a 1,500 rpm-centrifugation for 5 minutes at room temperature. To this purpose pellets of untreated OAW42 and OAW42Mer cells were resuspended in 0.9 ml of complete growth medium, then 0.1 ml of a 50 μM valinomycin solution (final concentration = 5 μM) were added and cells were placed at 37°C. After 30 minutes cells were collected by a 1,500 rpm-centrifugation for 5 minutes at room temperature, washed twice with 1 ml of PBS and pelleted again.

Pellets of untreated, drug-treated and valinomycin-treated cells were carefully resuspended in 0.9 ml of complete growth medium, then 0.1 ml of a 100 μg/ml JC-1 (final concentration = 10 μg/ml) solution were added and samples were incubated at 37°C in the dark. After 15 minutes, cells were centrifuged at 1,500 rpm for 5 minutes at room temperature and, after the removal of JC-1-containing medium, samples were washed once with 1 ml of PBS, resuspended in 1 ml of PBS and analysed by flow cytometry.

A FACScan flow cytometer was used to analyse a minimum of 30,000 cells per sample. Data were acquired in list mode and evaluated using Lysis II software (Becton Dickinson). Forward and side scatter were used to gate the viable population of cells. JC-1 monomers emit at 527 nm wave length (FL-1 channel, green range of visible light) whereas JC1-aggregates emit at 590 nm wave length (FL-2 channel, orange range of visible light). Duplicate samples of untreated control cells were used for compensation (FL-1 – FL-2) and flow cytometry profiles from these cells defined the 590 nm cut-off for drug treated samples.

3.2.13.2 Evaluation of apoptotic morphology by fluorescent microscopy

Untreated and cisplatin- and BBR 3464-treated OAW42 and OAW42Mer cells (0.5 x 10^6 cells) harvested at 48 and 72 hours from the end of treatment (T_{48} and T_{72}), were collected by a 1,500 rpm-centrifugation for 5 minutes at room temperature and stained with solution A (1 ml for 1 x 10^6 cells) as described in the 3.2.12.1 section. An aliquot of 30,000 stained cells was cytocentrifuged onto a glass slide for 3 minutes at 500 rpm (Cytospin 3) (Shandon Italia-Milan, Italy) at room temperature and, after that the cover slide was placed on the top of cells, glass slides were observed under fluorescence microscopy (excitation wave length = 588 nm, emission wave length = 610 nm) by using Zeiss fluorescence miscroscope (Carl Zeiss Italia-Arese, Italy). The percentage of apoptotic cells was determined by scoring at least 500 cells on each sample.

3.2.13.3 Evaluation of the genomic DNA fragmentation

The accumulation of DNA oligonucleosomal fragments was evaluated by a specific 'DNA agarose gel electrophoresis' assay essentially as described by Orlandi et al. (2001).

3.2.13.3.1 Preparation of a positive control of apoptosis induction

After harvesting in logarithmic growth phase, 3 x 10^6 HL-60 cells were seeded in 75-cm^2 flask with 20 ml of fresh growth medium and were then incubated at 37°C in a 5% CO_2 humidified atmosphere in air incubator for 24 hours.

Immediately before treatment, the 15 mM teniposide stock solution was diluted in growth medium to prepare a 11x solution (330 µM) and 2 ml of such 11x teniposide solution were added to the flask. After a 8-hour incubation at 37°C in a 5% CO_2 humidified atmosphere in air incubator, cells were pelleted by a 5-minute centrifugation at room temperature for 5 minutes.

After 2 PBS washes, cell pellets were resuspended in 1 ml of PBS, transferred in the 1.5 ml conical tubes and, single-cell suspensions were checked under the microscope and counted in a particle counter by mixing a small aliquot of cell suspension (generally 50 µl) with isoton solution.

Aliquots of 3 x 10^6 cells were collected by a 12,000 rpm-centrifugation at 4°C for 30 seconds and, after PBS removal, were frozen by snap freezing in liquid nitrogen and stored at –80°C until they were used for genomic DNA extraction.

3.2.13.3.2 Cellular DNA extraction

Frozen OAW42 and OAW42Mer cell pellets (3 x 10^6 cells), obtained from untreated and cisplatin- and BBR 3464-treated samples at 48 and 72 hours from the end of treatment (T_{48} and T_{72}), experimental points and the same amount of frozen teniposide-treated HL-60 cells were resuspended in 1 ml of lysis buffer [10 mM EDTA pH 8, 5 mM Tris-HCl pH 8, 0.5% (v/v) Triton X-100 (Sigma)] and incubated on ice for 30 minutes. Samples were centrifuged at 12,000 rpm for 30 minutes at 4°C then supernatant, containing low molecular weight DNA, was separated from the pellet, corresponding to high molecular weight DNA, and transferred in a 15 ml conical tube. Low molecular weight DNA samples were first digested for 1 hour at 37°C by addition of 0.5 ml of a 1,500 units/ml RNase A solution (final concentration = 500 units/ml). Then, 0.5 ml of the digestion solution [4% (w/v) SDS, 2 mg/ml proteinase K] were added and samples were incubated for 3 hours at 50°C. At the end of incubation, 2 ml of 1x TE pH 8-saturated phenol solution (Sigma) were added to each sample and tubes were centrifuged at 4,000 rpm for 5 minutes at 4°C. Supernatant aqueous phases were carefully removed and transferred in 15 ml tubes and 2 ml of 1x TE pH 8-saturated phenol/chloroform/isoamyl alcohol [25:24:1 (v/v/v)] solution were added. Samples were then centrifuged at 4,000 rpm for 5 minutes at 4°C and 1.8 ml of supernatant aqueous phase was carefully transferred in a 15 ml tube. Two hundred µl of 3 M sodium acetate pH 5.2 (final concentration = 0.3 M) and 5 ml (2.5 volumes) of absolute ethanol were added to each DNA sample and, tubes were centrifuged at 4,000

rpm for 5 minutes at 4°C. DNA pellets were then washed with 5 ml of 70% (v/v) ethanol, air dried and dissolved over night at 4°C in 1x TE pH 8.

3.2.13.3.3 Agarose gel electrophoresis

Purified-low molecular weight cellular DNA obtained from 3 x 10^6 cells was mixed with 4 µl of 6x gel loading buffer (final concentration = 1x) and loaded onto a 35 centimetres-length/20 centimetres-wide 1.5% (w/v) agarose gel prepared in 1x TBE buffer together with 3 µg of 1Kb DNA ladder marker (Sigma). Electrophoretic separation was performed by using 1x TBE as running buffer at a constant voltage of 50 volts for 5 hours at room temperature. Agarose gels were stained with ethidium bromide (10 µg/ml ethidium bromide, 1x TBE buffer) for 20 minutes onto a shaking platform and, after a 20-minute destaining in 1X TBE buffer on a shaking platform, the resultant DNA ladder was visualised and photographed under the UV transilluminator.

3.2.13.4 Western blot analysis

3.2.13.4.1 Protein extract preparation

Frozen OAW42 and OAW42Mer cell pellets (3 x 10^6 cells) obtained from untreated and cisplatin- and BBR 3464-treated samples at 24, 48 and 72 hours from the end of drug treatment (T_{24}, T_{48}, and T_{72}) were lysed on ice by gentle resuspension in 200 µl of ice-cold RIPA buffer (20 mM Tris-HCl pH 7.4, 150

mM NaCl, 5 mM NaF (Sigma), 2 mM PMSF and 10 μg/ml of the protease inhibitors aprotinin, leupeptin and pepstain). Tubes containing lysates were centrifuged at 12,000 rpm for 20 minutes at 4°C. Supernatants were then separated from pellet debris and transferred in 0.5 ml tubes. Protein content was quantified as described in the 3.1.4 section and protein extracts were stored at –80°C as 100 μg aliquots.

3.2.13.4.2 SDS PAGE

Protein extract fractionation was performed exactly as described in the 3.2.3.1 section with the only exception that a 12% polyacrylamide resolving gel was used.

3.2.13.4.3 Transfer of fractionated proteins from SDS-polyacrylamide gel to nitrocellulose filter

SDS PAGE-fractionated proteins were electroblotted onto nitrocellulose membrane using the same procedure described in the 3.2.3.2 section.

3.2.13.4.4 Immunological detection of nitrocellulose-immobilised proteins

Immunological detection of p53 (53 KDa), $p21^{waf1}$ (21 KDa), Bax (21 KDa), Bcl-2 (25 KDa), cyclin B_1 (62 KDa), $p34^{cdc2}$ (also named cdk 1) (34 KDa) and lamin B (66 KDa for full-length product, 45 KDa for cleavage product) proteins was carried out as described in the 3.2.3.3 section using the primary monoclonal or polyclonal antibodies anti-p53, anti-Bax, anti-Bcl-2, anti cyclin

B_1, anti-cdk1, anti-lamin B (Santa Cruz Biotechnology) and anti-$p21^{waf1}$ (Oncogene Science, Cambridge, MA, USA) at final concentrations of 1 μg/ml, respectively, and antimouse or antirabbit Ig horseradish peroxidase-linked whole antibodies diluted 1:2,500 as secondary antibodies.

To ensure equal loading of proteins on the gel, the monoclonal anti-tubulin β antibody (final concentration = 1 μg/ml) was used to detect the levels of such a protein on each blot.

To probe the same filter with alternative antisera, the probed membranes were immersed in a 50 ml tube containing 20-40 ml of 50°C-heated stripping solution (100 mM β-mercaptoethanol (Sigma), 2% (w/v) SDS, 62.5 mM Tris-HCl pH 6.7) and incubated for 30 minutes at 50°C. Stripped filters were washed twice with large volumes of T-PBS wash solution for 20 minutes at room temperature, then the procedure for immunological detection described in the 3.2.3.3 section was started.

3.2.14 *in vitro* cyclin B_1-associated cdk1 kinase activity assay

The kinase activity of the cyclin B_1-cdk1 complexes extracted from OAW42 and OAW42Mer after drug treatment was assessed *in vitro* as described by Orlandi et al. (2001).

3.2.14.1 Protein extraction

Frozen OAW42 and OAW42Mer cell pellets (1 x 10^6 cells) obtained from untreated and cisplatin- and BBR 3464-treated samples at 24, 48 and 72 hours

from the end of treatment (T_{24}, T_{48}, and T_{72}) were lysed on ice by gentle resuspension in 500 µl of ice-cold lysis buffer for kinase assay [1% (v/v) Nonidet P-40, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM AEBSF (Sigma), 1% (w/v) BSA prepared in PBS]. Cell lysates were clarified by a 15,000 rpm-centrifugation for 30 minutes at 4°C then the containing-protein supernatant was transferred in a 1.5 ml tube and placed on ice.

3.2.14.2 Immunoprecipitation

Five hundred µl of cellular lysate were mixed with 3 µg of the mouse monoclonal anti-cyclin B_1 antibody (Santa Cruz) and 100 µl of a 20% (v/v) protein A-Sepharose slurry (Amersham-Pharmacia Biotech.). The formation of immune complexes was carried out by a 4°C-incubation on a shaking platform. The protein A-Sepharose/anti-cyclin B_1 antibody/ cyclin B_1-cdk1 ternary immune complexes were then collected to the bottom of the tube by a 12,000 rpm-centrifugation for 2 minutes at 4°C. After the supernatant phase removal, immune complexes pellets were washed twice with 100 µl of lysis buffer for kinase assay and then twice with the same volume of lysis buffer for kinase assay minus BSA.

3.2.14.3 Kinase activity assay

Ten µl of 2x kinase buffer [40 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$ (Sigma), 10 µM ATP (Sigma)], 8 µl of a 375 ng/µl (3 µg) solution of histone H1 (Boehringer Mannheim Italia-Monza, Italy), 10 µCi of [γ-^{32}P]-ATP (3,000

Ci/mmole) and distilled water to a final volume of 20 µl were added to each immune complex pellet. The tubes were incubated at 30°C in a hot-bonnet thermal cycler for 20 minutes, then the reaction was stopped by adding an equal volume (20 µl) of 2x SDS loading buffer. Before loading onto SDS PAGE, samples were heated at 100°C for 5 minutes and then placed on ice for an additional 5 minutes.

3.2.14.4 SDS PAGE

SDS-polyacrylamide gel electrophoresis was carried out exactly as described in the 3.2.12.4.2 section. At the end of the run, and after the separation of glass plates, the stacking gel was removed by means of a razor blade whereas the resolving gel was transferred onto a plastic film, covered on the top with a sheet of Saran Wrap, placed onto a Hypercassette without intensifying screens and autoradiographed for different times at room temperature (short exposure times) or at 4°C (long exposure times) by using X-ray films.

Bands corresponding to the cyclin B_1-cdk1 kinase activity of the different samples were quantified by densitometric analysis. Kinase activities of untreated control and drug-treated samples were normalised on the number of cells in the G_2-M compartment and expressed in arbitrary densitometric units.

RESULTS

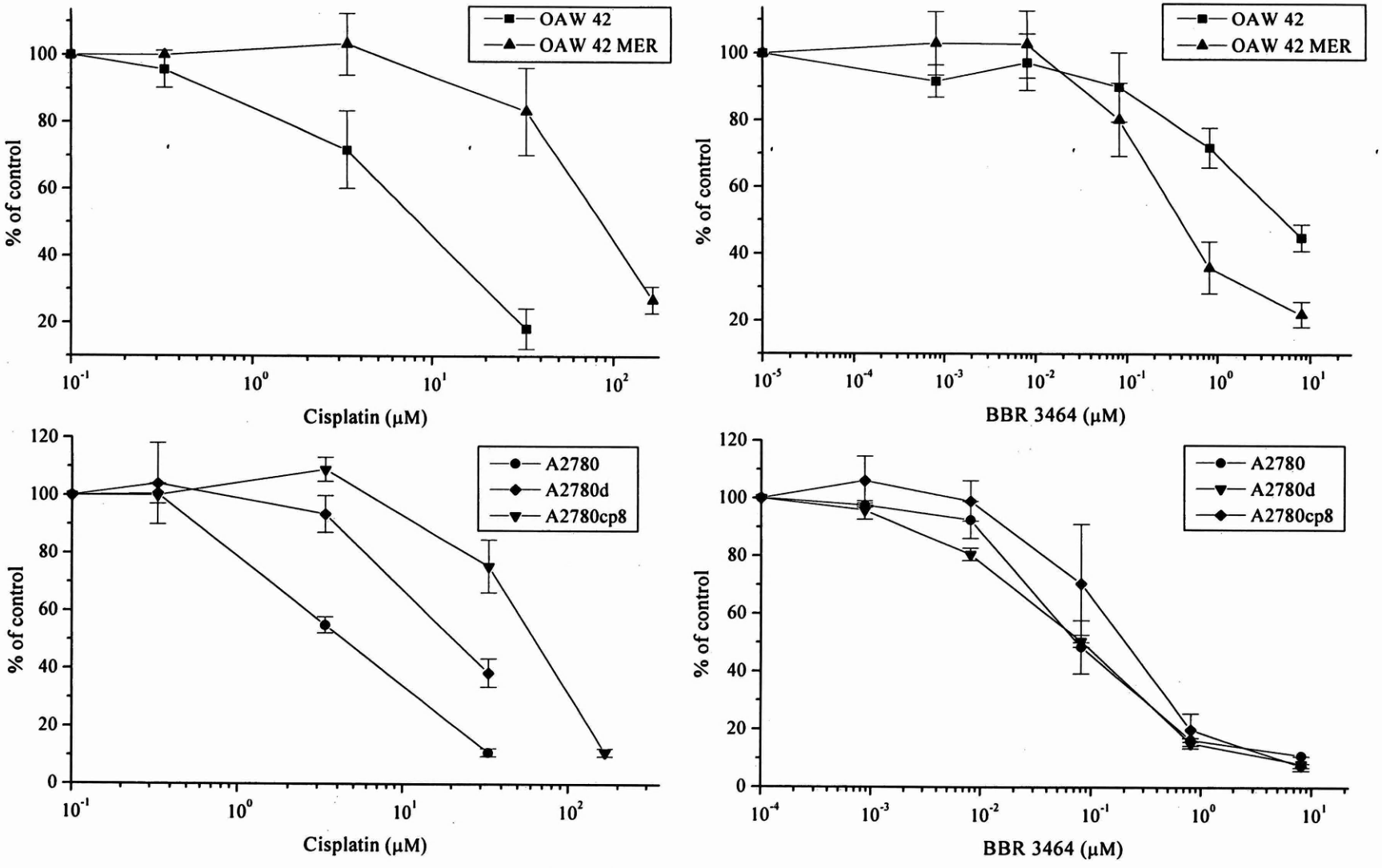
(4)

4.1 Cytotoxicity of cisplatin and BBR 3464

The cytotoxic activity of BBR 3464 was studied in comparison to cisplatin in a panel of human ovarian cancer cell lines by means of the growth inhibition assay. Cells were exposed to increasing concentrations of the two agents for 1 hour and the effect of the drugs was determined as variation in the cell number of treated samples (with respect to untreated control samples) after an additional 72 hours in drug-free medium. The concentrations of cisplatin and BBR 3464 able to inhibit cellular proliferation by 50% (IC_{50}) were extrapolated from the dose-effect curves.

A very interesting behaviour was found in the OAW42/OAW42Mer cell line pair, one sensitive and one with experimentally induced resistance to cisplatin, in which an opposite pattern of sensitivity to the two drugs was observed (Fig. 23 and Tab. IV). In fact, in respect of the parental-cisplatin sensitive OAW42 cell line, OAW42Mer cells were 10 times less sensitive to cisplatin but 14 times more sensitive to the novel trinuclear platinum complex BBR 3464. The pronounced collateral sensitivity of BBR 3464 seemed to be specific for this cell line pair. In fact, results obtained in a second pair of human ovarian carcinoma cell lines, A2780 and A2780cp8, one sensitive and one with experimentally induced resistance to cisplatin, indicated that a certain degree of cross-resistance between cisplatin and BBR 3464 was present, although the resistance index for the trinuclear platinum in the A2780cp8 was much lower than observed for cisplatin (2.5 vs 14-fold) (Tab. IV).

Figure 23. Sensitivity of OAW42, OAW42Mer, A2780, A2780d and A2780cp8 cells to cisplatin and BBR 3464 (1 hour exposure). The sensitivity was assessed by the growth inhibition assay. Cells were counted 72 hours after drug removal. Values represent the mean (± S.D.) of three independent experiments.


OAW 42
OAW 42 MER
% of control
Cisplatin (μM)
BBR 3464 (μM)
A2780
A2780d
A2780cp8

-Figure 23-

Table IV. Cytotoxic activity of cisplatin and BBR 3464 in human ovarian cancer cell lines. Drug activity is expressed in terms of concentration able to inhibit cell growth by 50% (IC_{50}). Values represent the mean (± S.D.) of three independent experiments. The resistance factor is the ratio of IC_{50} OAW42Mer/IC_{50} OAW42 or IC_{50} A2780d/IC_{50} A2780 or IC_{50} A2780cp8/IC_{50} A2780.

Cell line	Cisplatin		BBR 3464	
	IC_{50} (μM)	Resistance factor	IC_{50} (μM)	Resistance factor
OAW42	8.3 ±2.8	1.0	5.20 ±1.3	1.0
OAW42Mer	83.0 ±18.6	10.0	0.36 ±0.14	0.07
A2780	4.3 ±0.34	1.0	0.08 ±0.008	1.0
A2780d	20 ±3.25	4.7	0.077±0.03	0.96
A2780cp8	60.0 ±5.6	14.0	0.20 ±0.095	2.5

-Table IV-

The sensitivity profile to the two platinum-based anticancer agents was also determined in the A2780d cell clone which was selected in culture without drug exposure and characterised by alterations of the mismatch repair pathway (see section 4.2). Results obtained in the growth inhibition assay indicated that, in comparison to the parental cisplatin-sensitive A2780 cells, this cell clone was characterised by a moderate degree of resistance to cisplatin but it maintained the same sensitivity to the novel trinuclear platinum complex BBR 3464 (cisplatin IC_{50} for A2780 cells = 4.3±0.34 μM, cisplatin IC_{50} for A2780d cells = 20±3.25 μM; BBR 3464 IC_{50} for A2780 cells = 0.08±0.008 μM, BBR 3464 IC_{50} for A2780d cells = 0.077±0.03 μM).

4.2 Status of mismatch repair (MMR) and nucleotide excision repair (NER) systems in the cellular models

To verify whether the cytotoxic activity of BBR 3464 and cisplatin in our cell lines was influenced by the functional status of two of the most important DNA repair pathways, intracellular levels of the MMR enzymes, hMSH2, hMLH1, hPMS2, and of the NER enzyme ERCC1 were assessed by western blot analysis. In addition, microsatellite instability analysis was performed to ascertain the functional status of MMR. Again, RT-PCR experiments were carried out to evaluate ERCC1 gene expression and the molecular ratio between 'alternatively-spliced form' lacking exon VIII (ΔexVIII) and 'full-length form' of ERCC1 transcript.

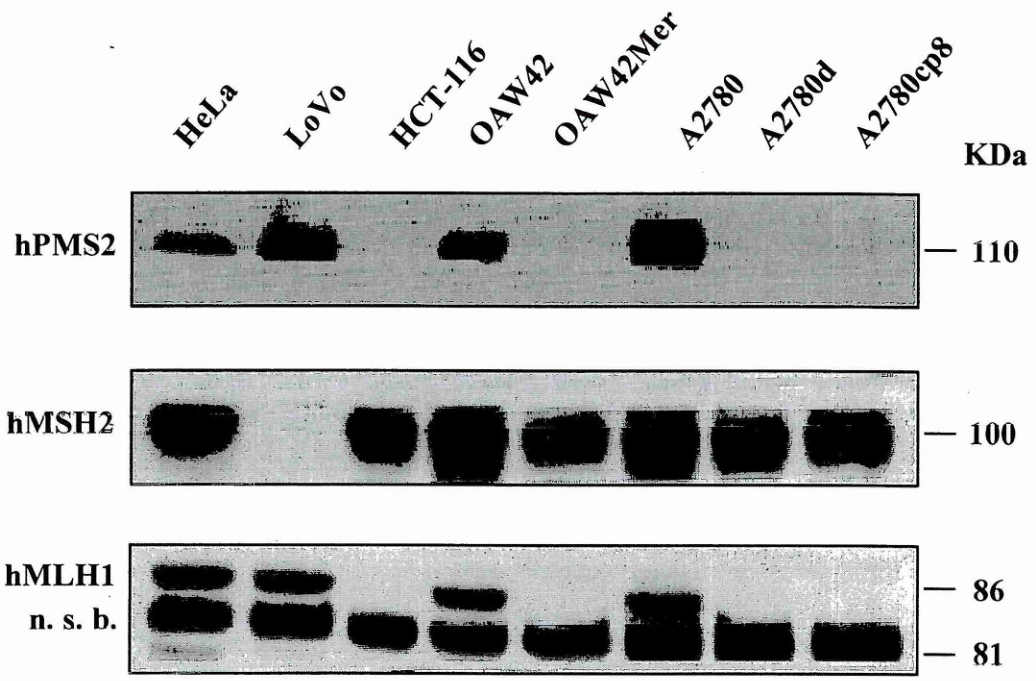
4.2.1 MMR

In western blotting experiments, nuclear extract from HeLa cells was used as a positive control for expression of the three MMR proteins hMLH1, hPMS2 and hMSH2, nuclear extract from LoVo cells line was used as negative control lacking hMSH2 expression, and nuclear extract from HCT-116 cells was used as negative control lacking the expression of both hMLH1 and hPMS2 proteins (Fig. 24).

As regards the 100-KDa hMSH2 protein, data obtained after immunostaining indicated that it was expressed in all the tested ovarian cancer cell lines. A different pattern of expression was observed for both 86-KDa hMLH1 and 110-KDa hPMS2 proteins. In fact, a lack of expression of the two human MutL homologues was observed in the cisplatin-resistant OAW42, A2780d and A2780cp8 cell lines, whereas these proteins were expressed in the parental cisplatin-sensitive counterparts OAW42 and A2780 cell lines (Fig. 24). It is important to underline that these differences in the expression of the three MMR proteins among the five cell lines were not due to a different loading of the gel, as demonstrated by the comparable intensity of an 81 KDa-non specific band.

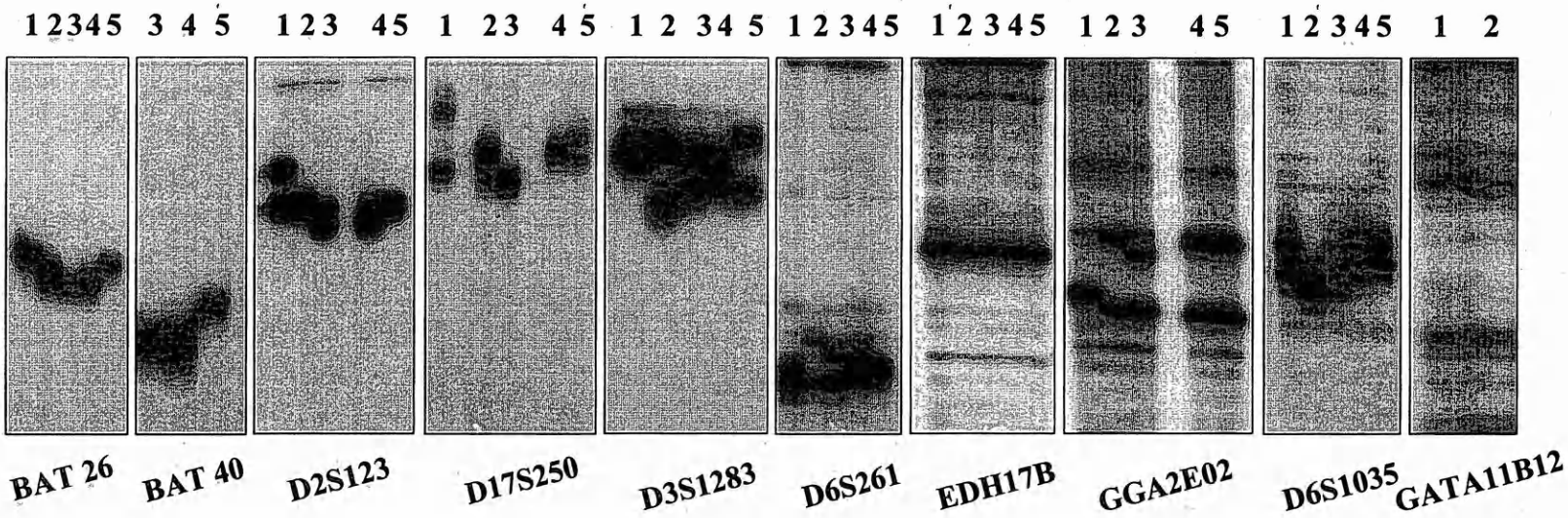
To verify whether the lack of such proteins was responsible for a functional inactivation of the MMR machinery in the cisplatin-resistant cells, the MMR efficiency was assessed in the five cell lines by means of the microsatellite instability assay at nine different loci (Fig. 25). These loci were selected on the basis of data previously published which indicate such microsatellite sequences

Figure 24. Structural analysis of the DNA mismatch repair (MMR) system by Western blotting assay of hPMS2, hMSH2 and hMLH1 protein expression in OAW42, OAW42Mer, A2780, A2780d and A2780cp8 cells. HeLa, LoVo and HCT-116 cell lines were used as specific controls for MMR protein expression. The non-specific lower band in the hMLH1 blot (n.s.b.) represented a control of protein loading.


HeLa
LoVo
HCT-116
OAW42
OAW42Mer
A2780
A2780d
A2780cp8
KDa
hPMS2
110
hMSH2
100
hMLH1
n. s. b.
86
81

-Figure 24-

Figure 25. Functional analysis of the DNA mismatch repair (MMR) system by microsatellite instability assay in OAW42 **(1)**, OAW42Mer **(2)**, A2780d **(3)**, A2780cp8 **(4)** and A2780 **(5)** cell lines.


1 2 3 4 5
3 4 5
1 2 3
4 5
1
2 3
4 5
1 2
3 4 5
1 2 3 4 5
1 2 3 4 5
1 2 3
4 5
1 2 3 4 5
1
2
BAT 26
BAT 40
D2S123
D17S250
D3S1283
D6S261
EDH17B
GGA2E02
D6S1035
GATA11B12

-Figure 25-

as the most indicative for a deficiency in MMR activity in human ovarian cancer tissues. Results obtained from sequencing gels corroborated those obtained with western blot analysis (Tab. V). In fact, conversely from what was observed in the MMR-proficient/cisplatin sensitive OAW42 and A2780 cells, a marked instability of the microsatellite sequences at seven/eight of the nine loci considered (78-89%) was detected in cisplatin-resistant cell lines lacking the expression of hMLH1 and hPMS2 proteins (OAW42Mer, A2780cp8 and A2780d). Specifically, data obtained from the analysis of microsatellite sequences indicated that the electrophoretic pattern corresponding to OAW42Mer cells was markedly different from that obtained for OAW42 cells at BAT 26, D2S123, D17S250, D3S1283, D6S261, GGAA2E02, and D6S1035 loci, whereas no differences were found between the two lines for the microsatellite sequences corresponding to EDH17B and GATA11B12 loci (Fig. 25 and Tab V). Moreover, the electrophoretic pattern observed for the cisplatin resistant A2780d cell clone indicated a marked instability of the microsatellite sequences corresponding to BAT 26, BAT40, D2S123, D17S250, D3S1283, D6S261, GGAA2E02 and D6S1035 loci. Microsatellite instability at the same loci (with the only exception of GGAA2E02 locus) was also detected for the other cisplatin-resistant cell line A2780cp8 (Fig. 25 and Tab V).

Overall, data obtained from analysis of the structural and functional status of the MMR suggest that, while the loss of MMR activity is an important event in determining cellular resistance to cisplatin, the functional status of MMR does not seem to be involved in the cellular response to BBR 3464.

Table V. Summary of the microsatellite instability analysis data. Symbols: + = microsatellite instability detected; - = microsatellite instability undetected; N.E. = not evaluated locus.

Locus	OAW42Mer vs OAW42	A2780d vs A2780	A2780cp8 vs A2780
BAT26	+	+	+
BAT40	N.E.	+	+
D2S123	+	+	+
D17S250	+	+	+
D3S1283	+	+	+
D6S261	+	+	+
EDH17B	-	-	-
GGAA2E02	+	+	-
D6S1035	+	+	+
GATA11B12	-	N.E.	N.E.

-Table V-

4.2.2 NER

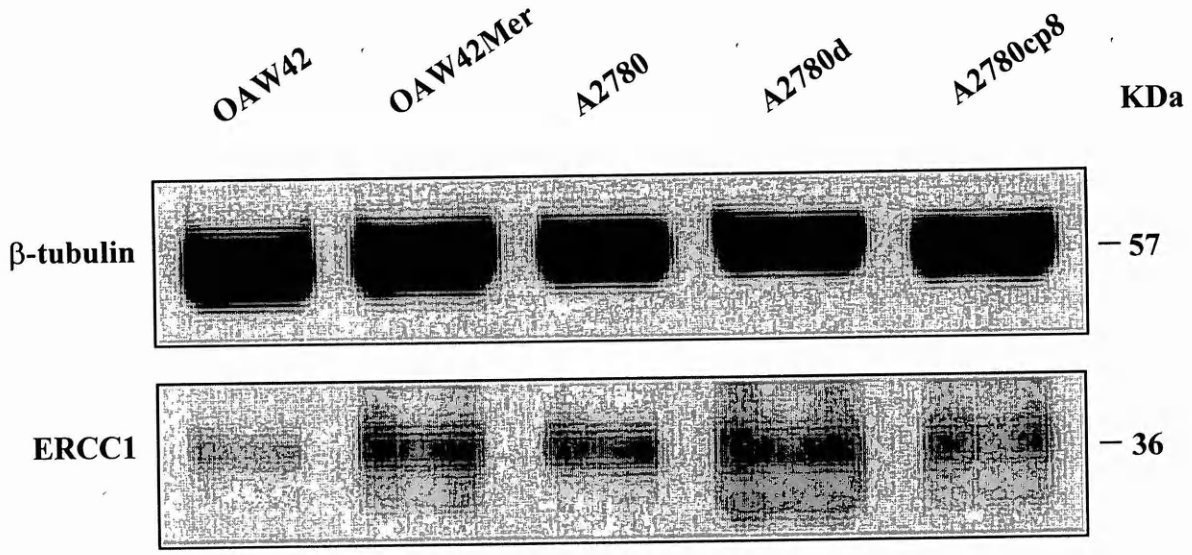
The intracellular mRNA and protein levels of the ERCC1 gene were evaluated by western blotting and quantitative non-competitive RT-PCR analyses, respectively, in OAW42, OAW42Mer, A2780, A2780d and A2780cp8 cell lines.

Results of western blot experiments indicated that this NER protein was present in all cell lines, although at a variable extent of expression. The amounts of ERCC1 enzyme present in the five cellular samples were then quantified by densitometric analysis and expressed as arbitrary densitometric units (a.d.u.), after normalisation with β-tubulin levels for the different gel loading.

As shown in Fig. 26, the level of the ERCC1 protein detected in the cisplatin-resistant OAW42Mer cell line was 3-fold higher than that observed in the OAW42 parental cell line (3.15 a.d.u and 1 a.d.u, respectively) (Tab. VI), whereas quite similar levels of ERCC1 expression were observed in A2780, A2780d and A2780cp8 cell lines (2.4, 2.9 and 1.9 a.d.u, respectively) (Tab. VI).

The results obtained in the quantitative non-competitive RT-PCR analysis (that was performed by using total RNA extracted from each cell line, a couple of primers able to select and amplify the 494 bp full-length ERCC1 mRNA molecule and a pair of primers that specifically amplify a 96 bp region of β-actin mRNA) corroborated the western blotting data. In fact, results from densitometric analysis of the gel photograph (Fig. 27) (expressed as arbitrary

Figure 26. Analysis of the DNA nucleotide excision repair (NER) system. Western blotting analysis of ERCC1 protein in OAW42, OAW42Mer, A2780, A2780d and A2780cp8 cells.


OAW42
OAW42Mer
A2780
A2780d
A2780cp8
KDa
β-tubulin
57
ERCC1
36

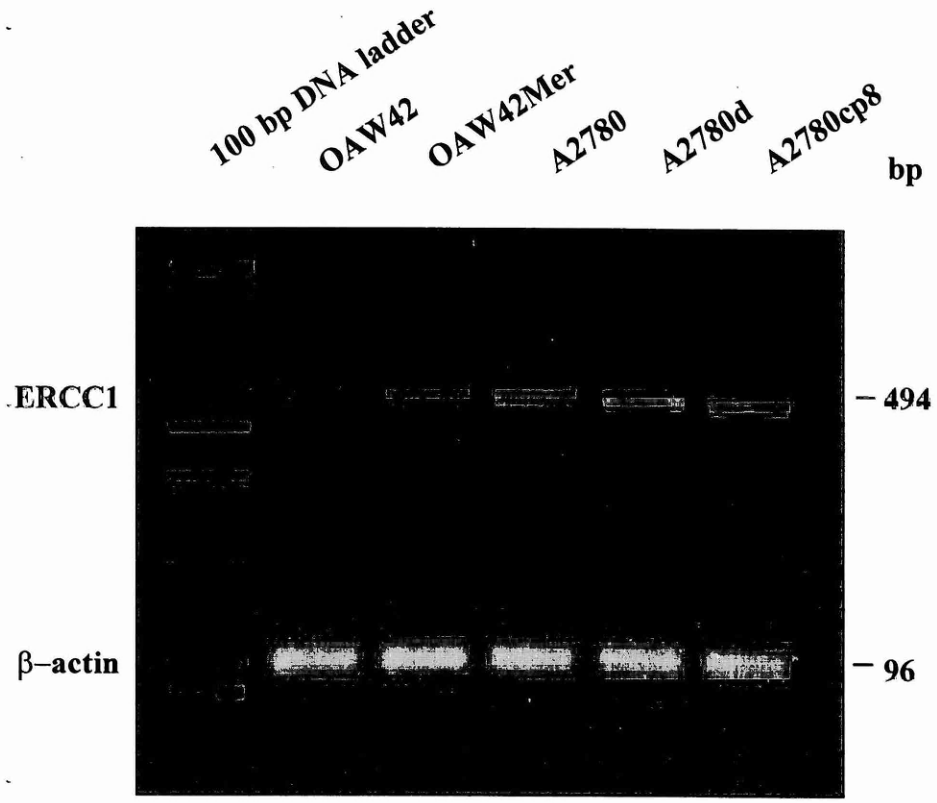
-Figure 26-

Table VI. Summary of the ERCC1 protein levels present in OAW42, OAW42Mer, A2780, A2780d and A2780cp8 cells [expressed as arbitrary densitometric units (a.d.u.)].

Cell lines	36 KDa ERCC1 (a.d.u.)
OAW42	1.0
OAW42Mer	3.15
A2780	2.4
A2780d	2.9
A2780cp8	1.9

-Table VI-

Figure 27. RT-PCR analysis of ERCC1 gene expression in OAW42, OAW42Mer, A2780, A2780d and A2780cp8 cell lines.


100 bp DNA ladder
OAW42
OAW42Mer
A2780
A2780d
A2780cp8
bp
ERCC1
β–actin
494
96

-Figure 27-

densitometric units after normalisation with β-actin levels for the different gel loading) indicated that ERCC1 mRNA levels present in the OAW42Mer cells were almost 3 times higher than those measured in the parental OAW42 cells (3.25 and 1 a.d.u, respectively) (Tab. VII), whereas a similar ERCC1 gene expression was detected in A2780, A2780d and A2780cp8 cells (3.3, 3.7 and 3.0 a.d.u, respectively) (Tab. VII). Moreover, results from this assay indicated that the up-regulation of ERCC1 expression in OAW42Mer cells occurred at the transcriptional or post-transcriptional level.

Since it has been reported that in cisplatin sensitive cell lines the low activity of the ERCC1 enzyme can be ascribable both to the downregulation of the ERCC1 gene expression (regulation at the transcriptional level) and to an increased ratio between alternatively-spliced (biologically inactive) and full-length (biologically active) forms of ERCC1 mRNA (regulation at post-transcriptional level), the amount of the alternatively-spliced ERCC1 mRNA form lacking exon VIII (ΔexVIII) was measured by a quantitative non competitive RT-PCR approach in our cellular models. Amplification reactions were carried out using a pair of primers able to select and amplify both full-length (267 bp) and ΔexVIII (196 bp) ERCC1 mRNA molecules together with a pair of primers that specifically amplify a 621 bp region of β-actin mRNA (Fig. 28).

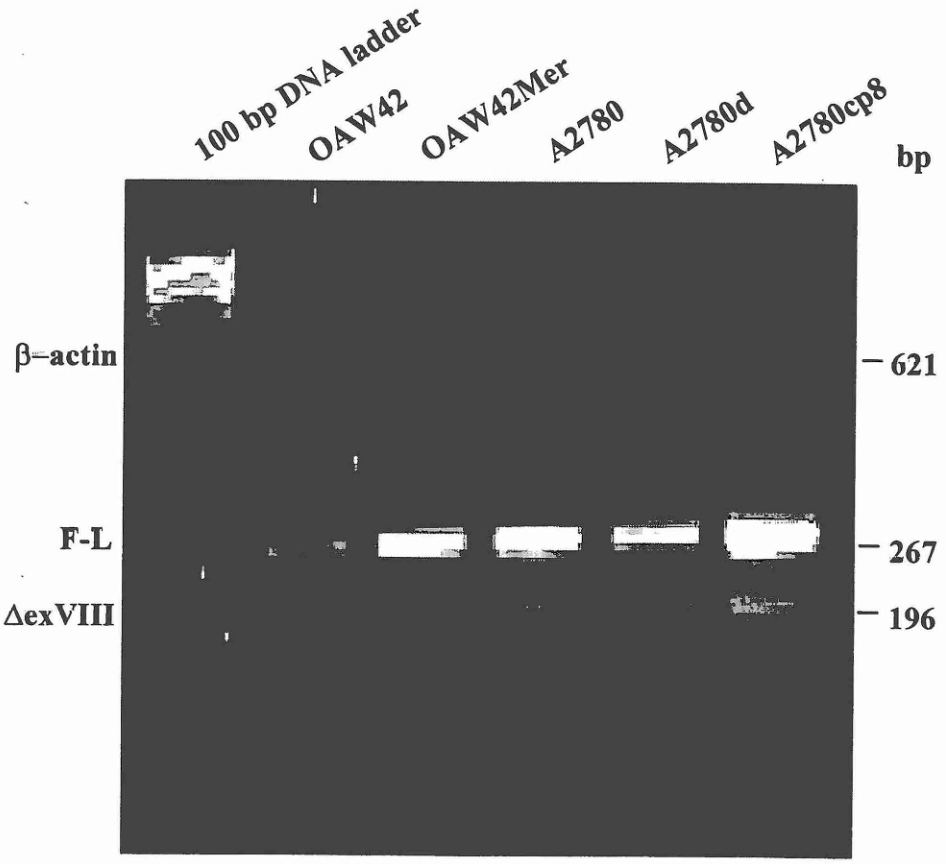
Results from the densitometric analysis of the gel photograph (Fig. 28) (expressed as arbitrary densitometric units after normalisation with β-actin levels for the different gel loading) (Tab. VIII) indicated that the amount of

Table VII. Summary of the ERCC1 gene expression levels (full-length mRNA) in OAW42, OAW42Mer, A2780, A2780d and A2780cp8 cells [expressed as arbitrary densitometric units (a.d.u.)].

Cell lines	494 bp ERCC1 (a.d.u.)
OAW42	1
OAW42Mer	3
A2780	3.6
A2780d	3
A2780cp8	3.7

-Table VII-

Figure 28. RT-PCR analysis of alternatively-spliced (ΔexVIII) and full-length (F-L) ERCC1 mRNA forms in OAW42, OAW42Mer, A2780, A2780d and A2780cp8 cell lines.


100 bp DNA ladder
OAW42
OAW42Mer
A2780
A2780d
A2780cp8
bp
β–actin
F-L
ΔexVIII
621
267
196

-Figure 28-

Table VIII. Summary of the ratios between alternatively-spliced (ΔexVIII) and full-length ERCC1 mRNA forms in OAW42, OAW42Mer, A2780, A2780d and A2780cp8.

Cell lines	ΔexVIII form/full-length form ratio
OAW42	0.322
OAW42Mer	0.1
A2780	0.213
A2780d	0.206
A2780cp8	0.285

-Table VIII-

ΔexVIII ERCC1 mRNA present in the parental OAW42 cells was greater than that detected in OAW42Mer cells, and the 'ΔexVIII form/full-length form' ratio in OAW42 cells was almost 3-fold higher than that found in OAW42Mer cells (OAW42Mer 'ΔexVIII form/full-length form' ratio = 0.1, OAW42 'ΔexVIII form/full-length form' ratio = 0.322) (Tab. VIII). Conversely, comparable 'ΔexVIII form/full-length form' ratios were detected in the A2780, A2780d and A2780 cp8 cell lines (ratios: 0.213, 0.206, 0.285, respectively) (Tab. VIII). Overall, our data suggest that the lower amount of ERCC1 enzyme found in the OAW42 cells compared to OAW42Mer cells, could be attributable to a high rate of post-transcriptional rearrangements as well as to a reduced transcription of the ERCC1 gene.

4.3 Evaluation of intracellular platinum accumulation

To verify whether the opposite pattern of sensitivity to cisplatin and BBR 3464 observed in the OAW42/OAW42Mer cell line pair, in comparison to the A2780/A2780cp8 cell line pair, can be explained in terms of a different accumulation of the two drugs in the cells, experiments were carried out to evaluate the intracellular platinum content in the different cell models after exposure to the two drugs. To this purpose, OAW42, OAW42Mer, A2780 and A2780cp8 cell lines were incubated for 1 hour with equimolar concentrations of cisplatin and BBR 3464 (0.3, 3.3 and 33.3 μM) and then intracellular

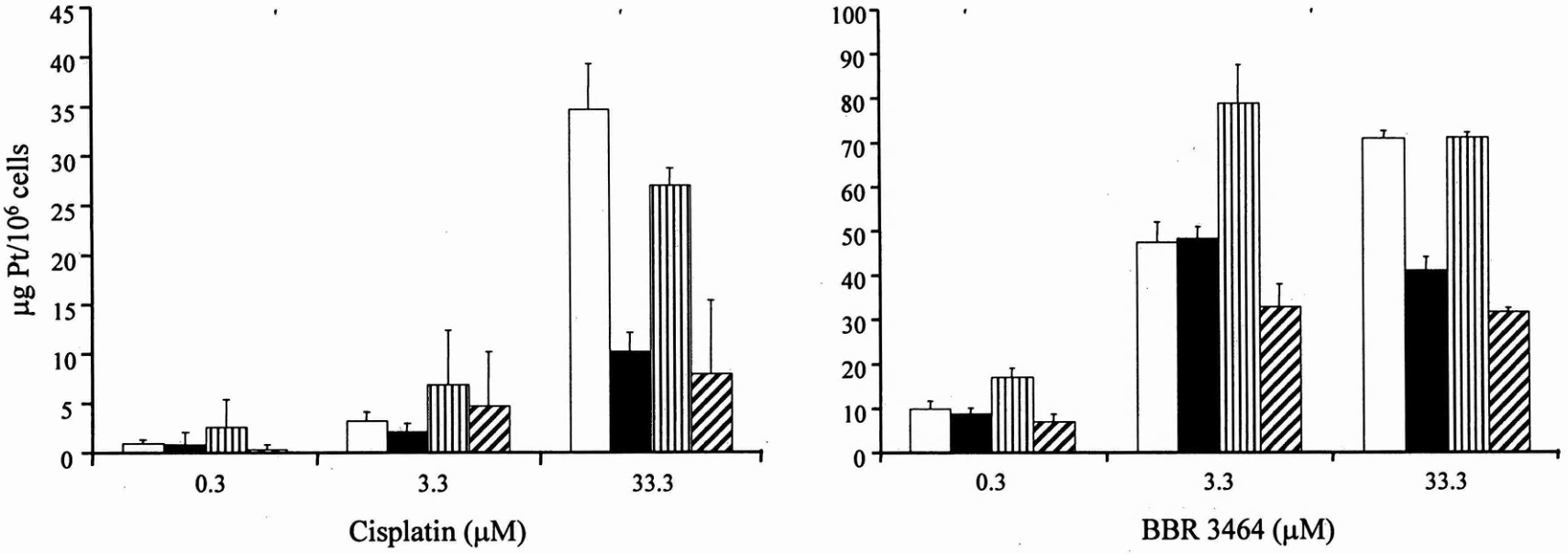
platinum content was measured by atomic absorption spectrometry and expressed as micrograms of platinum per 10^6 cells (μg Pt/10^6 cells).

The data obtained with this assay indicated that the accumulation of BBR 3464 was consistently higher than that of cisplatin. In fact, the quantity of platinum accumulated after exposure to the novel trinuclear platinum complex was from 2 to 20-times higher than that measured after treatment with cisplatin in the different cell lines (Fig. 29 and Tab. IX).

Data obtained after cisplatin treatment indicated that the levels of platinum found in the resistant OAW42Mer and A2780cp8 cells were lower than those detected in the corresponding parental sensitive OAW42 and A2780 cells at all the tested doses. Specifically, for the OAW42 and OAW42Mer cell line pair, the differences were minimal at the lower drug concentrations (Tab. IX) whereas at the highest drug concentration (33.3 μM) the amount of intracellular platinum content was significantly different ($p<0.05$, Student t test) between sensitive and resistant cells (Tab. IX). As regards the second pair of ovarian cell lines, the amount of platinum accumulated in the A2780 cells was significantly higher ($p<0.05$, Student t test) than that observed in A2780cp8 cells, at all cisplatin concentrations used, although the maximum difference was evident at the highest cisplatin dose (33.3 μM) (Tab. IX).

After exposure to different concentrations of BBR 3464, a lower platinum accumulation consistently was observed in the A2780cp8 cell line as compared to its parental counterpart A2780 cell line (Tab. IX). These differences were significant ($p<0.05$, Student t test) at the two highest BBR3464 concentrations

Figure 29. Cellular accumulation of platinum after a 1-hour exposure to cisplatin and BBR 3464 as assessed by atomic absorption spectrometry in OAW42 (□), OAW42Mer (■), A2780 (■) and A2780cp8 (■) cell lines. Values represent the mean (± S.D.) of three independent experiments.


µg Pt/10⁶ cells
45
40
35
30
25
20
15
10
5
0
0.3
3.3
33.3
Cisplatin (µM)
100
90
80
70
60
50
40
30
20
10
0
0.3
3.3
33.3
BBR 3464 (µM)

-Figure 29-

Table IX. Summary of the data regarding cellular accumulation of platinum in OAW42, OAW42Mer, A2780, and A2780cp8 cells. Data are expressed as micrograms of platinum per 10^6 cells (μg Pt/10^6 cells).

Cell lines	Cisplatin (μM)	μg Pt/10^6 cells	BBR 3464 (μM)	μg Pt/10^6 cells
OAW42	0.3	0.95±0.4	0.3	9.8±1.8
	3.3	3.23±0.9	3.3	47.4±4.6
	33.3	34.7±4.6	33.3	70.9±1.7
OAW42Mer	0.3	0.85±1.2	0.3	8.8±1.4
	3.3	2.1±0.9	3.3	48.2±2.9
	33.3	10.2±2	33.3	41.1±3.1
A2780	0.3	2.6±2.8	0.3	17±2.0
	3.3	6.85±5.5	3.3	78.9±8.7
	33.3	27±1.8	33.3	71.2±1.2
A2780cp8	0.3	0.35±0.5	0.3	6.8±2.0
	3.3	4.7±5.5	3.3	32.8±5.2
	33.3	7.9±7.5	33.3	31.7±1.0

-Table IX

Results obtained after exposure of OAW42 and OAW42Mer cells to the novel trinuclear platinum complex indicated that the amount of platinum accumulated in the two cell lines was similar at the concentration of 0.3 μM and 3.3 μM (Tab. IX). Conversely, after treatment with the highest BBR 3464 concentration, a significantly ($p<0.05$, Student t test) lower platinum accumulation was detected in OAW42Mer cells than in OAW42 cells (Tab. IX).

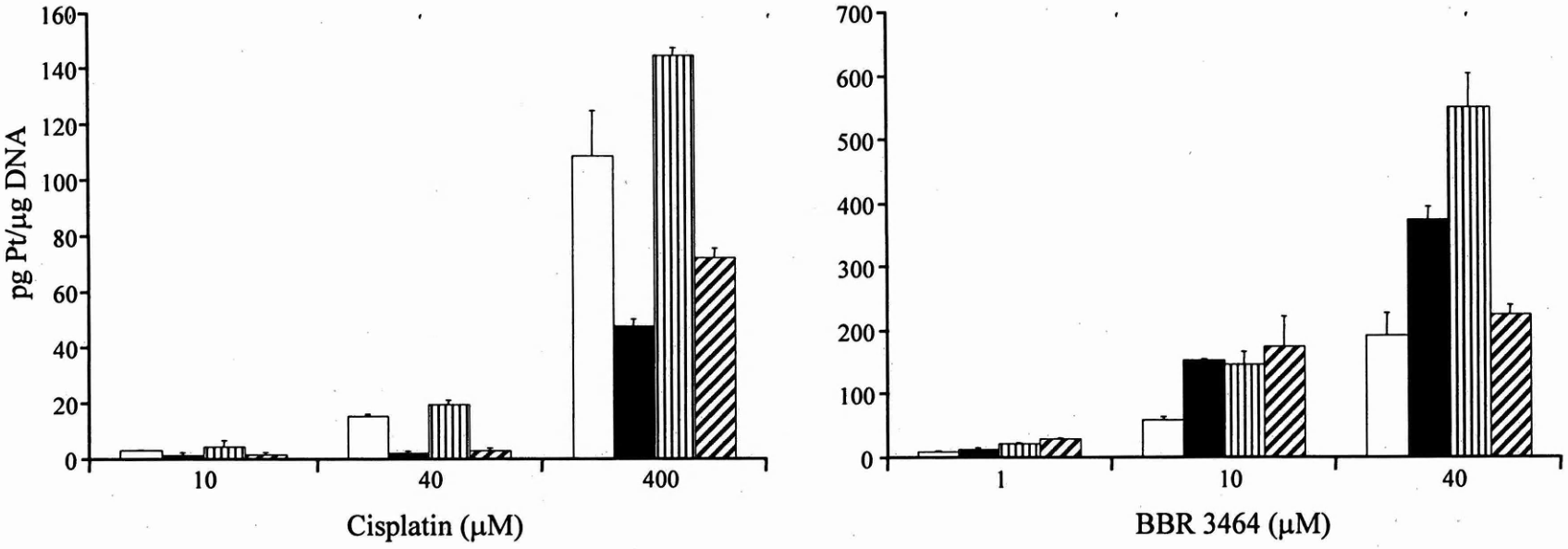
4.4 Evaluation of platinum bound to cellular DNA

Since genomic DNA has been described as the most important cellular target for both cisplatin and BBR 3464, we assessed whether the different activity profiles observed for the two drugs were due to a different capacity of the drugs to penetrate the nucleus and covalently bind cellular DNA.

The amount of platinum bound to DNA was measured by mass spectroscopy analysis following exposure of OAW42, OAW42Mer, A2780 and A2780cp8 intact cells to equimolar concentrations of the two platinum-based drugs (10, 40, 400 μM of cisplatin and 1, 10, 40 μM of BBR 3464), and it was expressed as picograms of platinum per micrograms of DNA (pg Pt/μg DNA).

Results obtained from such experiments showed that, after exposure to cisplatin, the extent of DNA platination found in resistant OAW42Mer and A2780cp8 cell lines was lower than that observed in sensitive cells at all drug concentrations used (Fig. 30 and Tab. X).

Figure 30. Platinum bound to genomic DNA after a 1-hour exposure to cisplatin and BBR 3464 as assessed by mass spectroscopy analysis in OAW42 (□), OAW42Mer (■), A2780 (■) and A2780cp8 (■) cell lines. Values represent the mean (± S.D.) of three independent experiments.


pg Pt/µg DNA
160
140
120
100
80
60
40
20
0
10
40
400
Cisplatin (µM)
700
600
500
400
300
200
100
0
1
10
40
BBR 3464 (µM)

-Figure 30-

Table X. Summary of the data regarding platinum bound to genomic DNA of in OAW42, OAW42Mer, A2780, and A2780cp8 cells. Data are expressed as picograms of platinum per micrograms of genomic DNA (pg Pt/μg DNA).

Cell lines	Cisplatin (μM)	pg Pt/μg DNA	BBR 3464 (μM)	pg Pt/μg DNA
OAW42	10	3.22±0.15	1	8.4±2.12
	40	15.2±0.8	10	59±5.0
	400	108.5±16.2	40	191.7±35
OAW42Mer	10	1.3±1.0	1	13±2.5
	40	1.8±1.0	10	152.6±2.4
	400	47.5±2.6	40	373.7±20.7
A2780	10	4.2±2.5	1	21±1.9
	40	19.5±1.5	10	145.5±21
	400	144.5±2.9	40	550.22±54
A2780cp8	10	1.5±0.8	1	28.7±1.4
	40	2.9±0.9	10	173.2±48.9
	400	72±3.6	40	225.1±15

-Table X-

After treatment with BBR 3464, significant ($p<0.05$, Student t test) lower levels of platinum bound to genomic DNA were found in the BBR 3464-resistant cells (OAW42) than in BBR 3464-sensitive cells (OAW42Mer) at all drug concentrations tested (Tab. X). These differences were constant, with a ratio between pg Pt/μg DNA of sensitive cells and pg Pt/μg DNA of resistant cells ≅ 2, at all BBR 3464 concentrations tested (Tab. X).

In the A2780 and A2780cp8 cell line pair, a similar trend was observed only at the highest BBR 3464 concentration (with a ratio between pg Pt/μg DNA of A2780 and pg Pt/μg DNA of A2780cp8 ≅ 2). In fact, at lower BBR 3464 concentrations, no marked difference between sensitive and resistant cells was detected (Tab. X).

Results from these experiments also indicated that the ability of the novel trinuclear platinum complex to covalently bind genomic DNA was greater than that of cisplatin. In fact, when we compared the extent of platinum bound to DNA after treatment of the four cell lines with 10 and 40 μM cisplatin to that measured after exposure of the cells to the same concentrations of BBR 3464, we found higher values (from 10 to 40 times), in terms of picograms of platinum bound per micrograms of DNA, for the novel trinuclear platinum complex.

4.5 Evaluation of interstrand cross-links formation

The ability of BBR 3464 to produce DNA interstrand cross-links *in vitro* in a dose-dependent manner was compared to that of cisplatin by using the naked pSP73 vector as substrate. This DNA plasmid was selected on the basis of its low content of guanines, which are the DNA nucleotides most frequently covalently attached by platinum-based drugs, in the attempt to reduce the possible background of the assay due to higher DNA packaging and/or fragmentation. Experiments were carried out by treating the DNA vector for 1 hour at 37°C in the dark with different concentrations of cisplatin or BBR 3464. Damage reactions were then stopped by ethanol precipitation, and treated and untreated samples, previously denatured by thermal treatment (with the exception of one of the two untreated control samples) were loaded onto an agarose gel. It is important to stress that the drug concentrations used in these experiments were selected on the basis of the results obtained from several preliminary experiments which were performed to identify the drug doses able to guarantee an optimal balance between dose-dependent induction of DNA interstrand cross-links and general background of the assay.

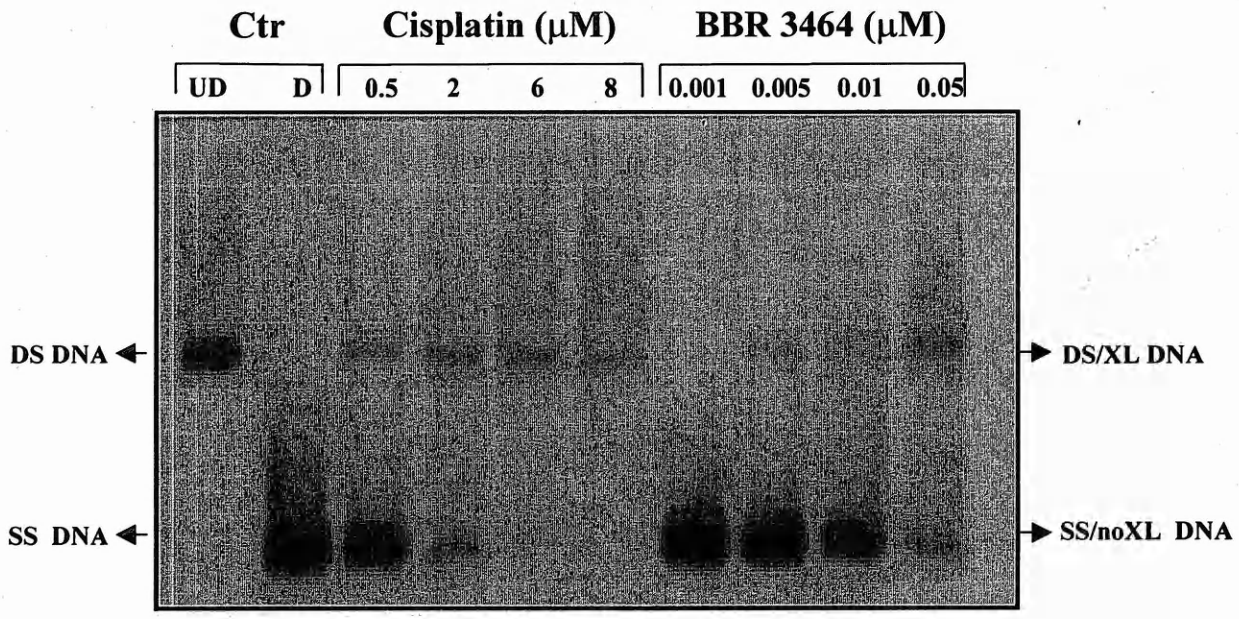
Figure 31 shows a representative agarose gel electrophoresis experiment of dose-dependent accumulation of drug-induced DNA interstrand cross-links. Untreated control samples, subjected or not subjected to heat denaturation before gel loading (CTR D and CTR UD respectively) are characterised by single DNA bands with different electrophoretic mobility. Specifically, the signal corresponding to the CTR UD sample, which consists of double-

stranded DNA molecules not exposed to the thermal treatment (DS DNA), is characterised by an electrophoretic mobility lower than that of the heat-denatured control sample (CTR D). The latter sample represents the sum of single-stranded plasmid DNA molecules (SS DNA).

In drug treated samples, which were all heat denatured before gel loading, it is possible to appreciate the appearance of a band (DS/XL DNA) characterised by an electrophoretic mobility similar to that of the DS DNA present in the CTR UD sample. In fact, this DS/XL DNA band represents the sum of double stranded plasmid molecules in which the strand separation induced by thermal treatment was inhibited by drug-induced formation of interstrand cross-links.

Results reported in figure 31 indicate that the intensity of the DS/XL DNA bands obtained after treatment with different concentrations of cisplatin or BBR 3464 does not increase in a dose-dependent manner. Conversely, it is possible to observe a dose-dependent decrease in the amount of the bands formed by single-stranded non cross-linked (SS/noXL) DNA molecules. These latter molecules are characterised by an electrophoretic mobility similar to that of the SS DNA band present in the CTR D samples. This particular behaviour can be explained by considering the fact that relatively high drug concentrations are able to induce several alterations in the pSP73 vector chemical-physical properties thus producing undesired collateral phenomena, such as elevated rates of DNA packaging and/or fragmentation, with a consequent increase of the gel background. To obtain quantitative information on the ability of BBR 3464 and cisplatin to form DNA interstrand cross-links, this methodological problem was circumvented by calculating indirectly the

Figure 31. Evaluation of interstrand cross-links induction on naked pSP73 vector after a 1-hour *in vitro* exposure to cisplatin and BBR 3464. Ctr UD = untreated control sample not subjected to thermal denaturation before loading; Ctr D = untreated control sample subjected to thermal denaturation before loading; DS DNA = double stranded DNA; SS DNA = single stranded DNA; DS/XL DNA = double stranded-cross-linked DNA; SS/noXL DNA = single stranded-not cross-linked DNA.


Ctr
Cisplatin (μM)
BBR 3464 (μM)
UD
D
0.5
2
6
8
0.001
0.005
0.01
0.05
DS DNA
SS DNA
DS/XL DNA
SS/noXL DNA

-Figure 31-

extent of such DNA lesions at the different drug concentrations on the basis of the disappearance of the SS/noXL DNA band in drug-treated samples with respect to the untreated control sample (CTR D).

Data obtained from densitometric analysis in cisplatin-treated samples (Tab. XI) indicate that after a 1-hour exposure, a considerable decrease (38%) of the SS/noXL DNA band intensity, due to the formation of DNA interstrand cross-link lesions, was already detectable at the lowest drug concentration (0.5 μM). The extent of DNA interstrand cross-links increased in a dose-dependent manner (58% and 99.3%, at 2 and 6 μM respectively) up to the highest drug concentration (8 μM) where an almost complete disappearance of the signal corresponding to the SS/noXL DNA band was observed.

After a 1-hour exposure of the pSP73 plasmid to BBR 3464 (Fig. 31), a dose-dependent decrease of the SS/noXL DNA band and a corresponding increase of the DS/XL DNA band were detected at drug concentrations up to 0.01 μM. Conversely, at the highest concentration of the trinuclear platinum complex (0.05 μM), the strong decrease of the SS/noXL DNA band was not accompanied by a correspondent increase of the DS/XL DNA band. However, it was indicative of an elevated extent of DNA interstrand cross-links formation (62%) (Tab. XI).

These results indicate that the ability of BBR 3464 to form *in vitro* DNA interstrand cross-links is quite similar to that of cisplatin. However, from a quantitative point of view, results indicate that the affinity of the novel trinuclear platinum compound for the DNA is significantly greater than that of

Table XI. Summary of data regarding interstrand cross-links induction on naked pSP73 vector. Data are expressed as percentage value of band signal in drug-treated samples with respect to untreated-heat denatured control sample.

Sample	Drug Concentrations (μM)	% vs CTR D
Untreated Control D	/	100
Treated cisplatin	0.5	62
Treated cisplatin	2	42
Treated cisplatin	6	0.7
Treated cisplatin	8	0
Treated BBR 3464	0.001	88
Treated BBR 3464	0.005	77
Treated BBR 3464	0.01	50
Treated BBR 3464	0.05	38

-Table XI-

cisplatin. In fact, the concentration of BBR 3464 necessary to produce on the plasmid DNA a number of interstrand cross-link lesions able to reduce the intensity of the SS/noXL DNA band of about 50%, was 140-times lower than that required for cisplatin (0.01 μM vs 1.4 μM). It is important to stress that these findings are in agreement with those obtained after measurement of platinum bound to genomic DNA after exposure of intact cells to the two drugs (section 4.4).

4.6 Sequence selectivity of adduct formation of BBR 3464 and cisplatin

In an attempt to qualitatively characterise the molecular lesions induced by BBR 3464 to DNA, the Taq stop assay was used for the evaluation of the sequence specificity of DNA adducts generated by the novel trinuclear platinum complex in comparison to that of cisplatin. The sequence specificity of damage induced by the two platinum-based compounds was evaluated on DNA template molecules characterised by an increasing structural complexity and in experimental conditions progressively closer to the physiological environment.

The first experiments of Taq stop assay were carried out on naked pSP73 plasmid DNA exposed to the two drugs *in vitro*. As already mentioned, such DNA vector represents an excellent substrate to investigate the effect of *in vitro* treatment with potent electrophilic intermediates, like cisplatin and BBR 3464, on the basis of its low content of possible nucleophilic targets such as guanine and/or adenine DNA nucleotides.

An opportunely selected amount of purified plasmid DNA was exposed *in vitro* to different concentrations of cisplatin and BBR 3464 for 2 hours at 37°C in the dark. Such drug concentrations were chosen on the basis of the results of preliminary experiments that were carried out to define the amount of cisplatin and BBR 3464 at which the single-hit kinetics (each DNA molecule receives at most one lesion) was achieved. Such kinetics (which represent an important parameter to be considered in order to obtain significant information from this technique) are satisfied when a clear dose-dependent increase in the signal of the bands (corresponding to the sites whose Taq DNA polymerase activity was prematurely halted by drug-induced DNA adducts) is observed.

Drug exposed DNA vector was used as template in linear PCR together with the ^{32}P-labelled Sp6 primer. The same primer was also utilised to perform pSP73 sequencing reactions by means of a PCR-based dideoxy method. Samples from Taq stop and sequencing reactions were then simultaneously loaded onto the sequencing gel and the sequence selectivity of the DNA lesions, generated by drug treatment, was exactly (±2 bp) pinpointed by comparing the position of the bands (representing the nucleotide at which Taq polymerase was stopped by the drug-generated adduct) and the dideoxy sequencing lanes.

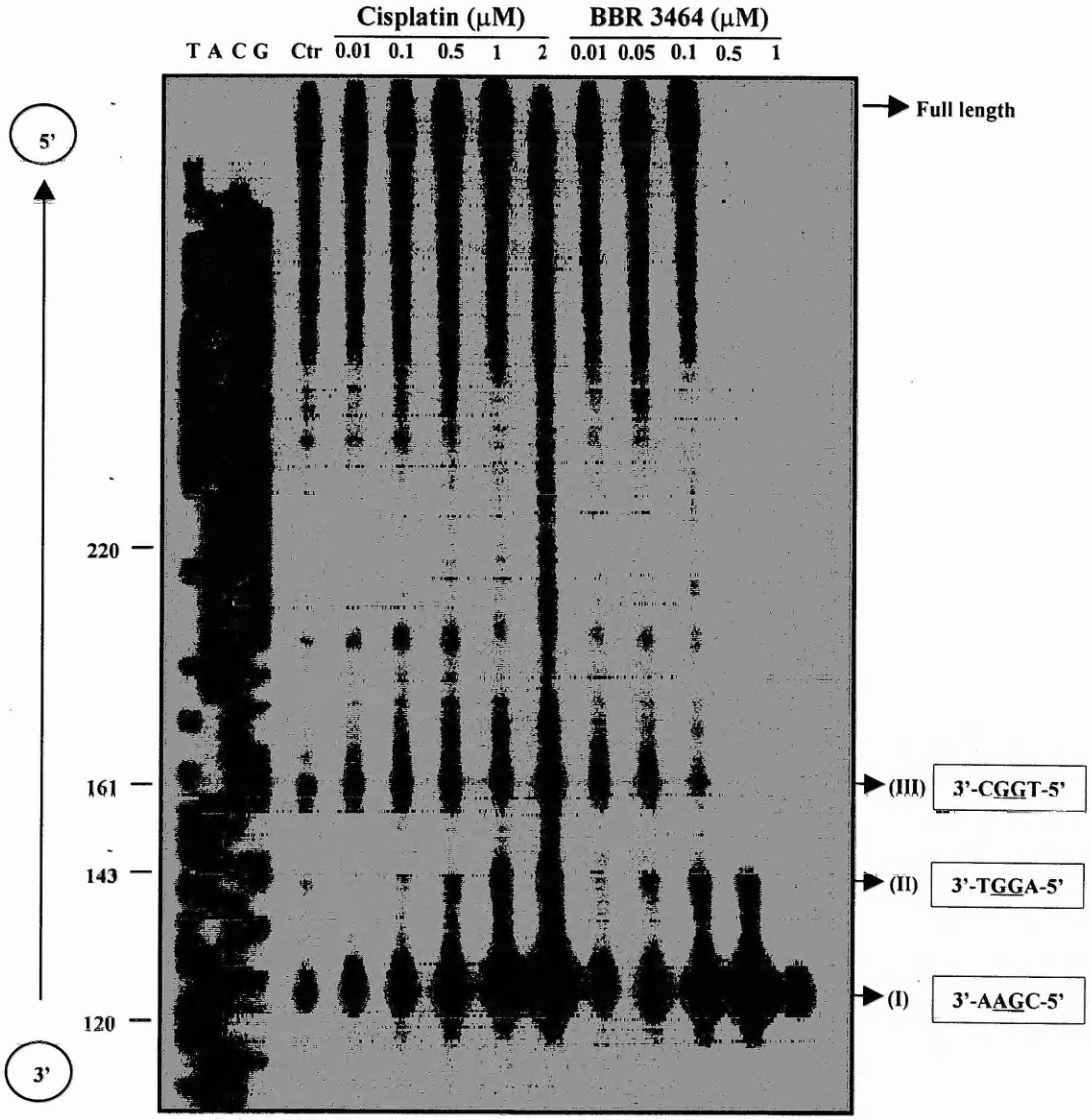
It is important to stress that the sequence selectivity of DNA lesions induced by the two platinum-based compounds on the plasmid was evaluated in a small region of the template. In fact, the labelled Sp6 primer was able to select and amplify (in a linear fashion) a selected ~190 bp portion of the pSP73 vector included between the primer annealing region and the artificial Taq stop site

generated by cutting the vector with the HaeII restriction endonuclease (Fig. 21).

Figure 32 shows a representative picture of a sequencing gel in which Taq stop and sequencing reactions, carried out on naked pSP73 plasmid DNA, were depicted. Results obtained with this assay indicate that the two platinum-based anticancer drugs share almost the same sequence preference of adduct formation. In fact, after a 2-hour exposure to various concentrations of each drug, the presence of three distinct bands (I, II and III) was clearly detectable in the polyacrylamide gel. These radioactive spots represent the pool of DNA fragments characterised by a number of base pairs lower than that present in the full-length fragment due to the premature stops of Taq DNA polymerase activity caused by the drug-induced lesions at the 3'-AG-5' (band I) and 3'-GG-5' (bands II and III) dinucleotide sites. The presence of weak bands at the same sites in the untreated DNA control sample was also detected. This phenomenon is thought to be a technical problem of the assay, as already reported by other authors (Bubley et al., 1994; Grimaldi et al., 1994b). In fact, such background signals seem to be generated by intrinsic obstructions to Taq polymerase, since they constitutively appear in the same positions and may be due to secondary structures of DNA blocking to some extent the progress of Taq polymerase in the first round of the PCR. However, all attempts to overcome the effects of such secondary structures, such as the inclusion of the co-solvent DMSO into the PCR mixtures, failed to remove these bands.

Results reported in Figure 32 indicate that the intensity of the three bands corresponding to the Taq stop sites generated by cisplatin is significantly

Figure 32. Detection of adducts induced by BBR 3464 and cisplatin in naked pSP73 vector after a 2-hour *in vitro* drug exposure. The sites of DNA lesions are indicated as I-III. The corresponding adduct sites on the DNA template (as determined from the consensus sequence) are underlined.


Cisplatin (μM)
BBR 3464 (μM)
T A C G
Ctr
0.01
0.1
0.5
1
2
0.01
0.05
0.1
0.5
1
Full length
5'
3'
220
161
143
120
(III)
3'-CGGT-5'
(II)
3'-TGGA-5'
(I)
3'-AAGC-5'

-Figure 32-

different. Specifically, the signal corresponding to the band I was greater than those corresponding to the bands II and II, thus suggesting that the number of lesions generated by cisplatin at the 3'-AG-5' dinucleotide on the 3'-AAGC-5' (band I) sequence was higher than those produced at 3'-GG-5' dinucleotides on 3'-TGGA-5' (band II) and 3'-CGGT-5' (band III) sequences. Moreover, results indicate that cisplatin affinity for the 3'-GG-5' site is greater when such a dinucleotide is located in the 3'-CGGT-5' (band III) instead of that in the 3'-TGGA-5' (band II) DNA sequence, thus suggesting that the affinity with which this drug is able to covalently bind the same target site (for example, the 3'-GG-5' dinucleotide) can also be influenced by neighbouring nucleotides.

Superimposable results were detected in BBR 3464-treated samples (Fig. 32) with the only difference being that a lack of dose-dependent increase in band intensities at sites II and III was found. This phenomenon can be explained by a lack of the single-hit kinetics (Hartley and Wyatt, 1997). In fact, as the drug concentration increases the percentage of template DNA molecules bearing more than one lesion will also increase and Taq polymerase will be blocked at the first lesion met (the one closest to the primer). This explanation seems to be applicable to the results obtained at the BBR 3464 concentrations of 0.1 and 0.5 μM. As regards the highest drug dose (1 μM), it is possible to assume that the extent of damage on plasmid DNA molecules was sufficient to completely inhibit linear PCR reactions, probably by preventing the annealing step between Sp6 primer and the template strand of the pSP73 vector (Fig. 32).

Although the Taq stop assay was not intended to quantify DNA adducts, results from the sequencing gel shown in Figure 32 clearly indicate that the

efficiency of BBR 3464 to generate DNA adducts was greater compared than that of cisplatin. In fact, by considering the results obtained at concentrations of the two platinum-based compounds at which the requirement of single-hit kinetics was satisfied, it is possible to observe that in order to generate bands of comparable intensity at the three sites, the required cisplatin dose is almost 2 times higher than that of BBR 3464.

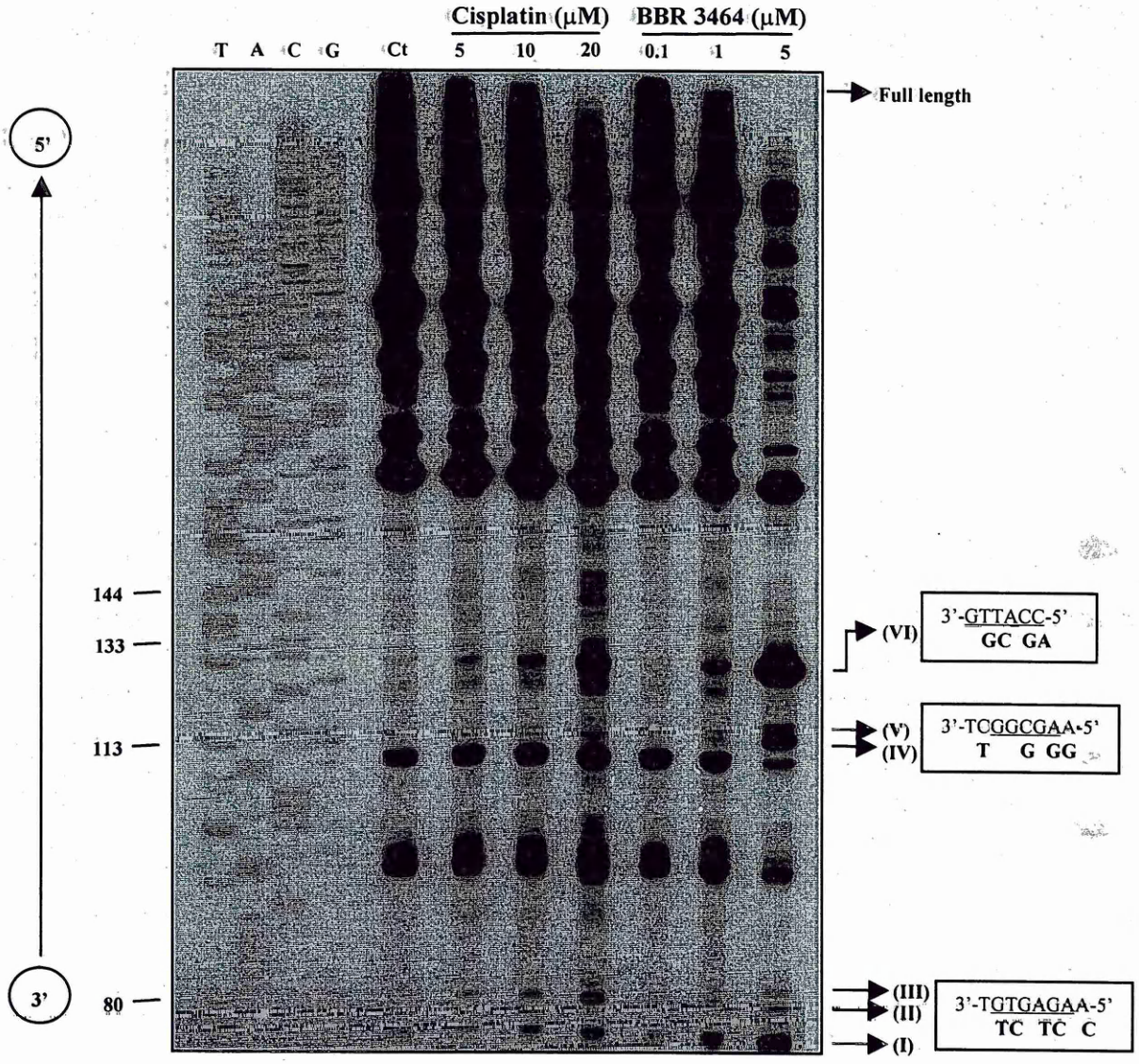
The sequence specificity of DNA lesions produced by the two platinum-based anticancer drugs was also evaluated on a more structurally complex DNA substrate. To this purpose, genomic DNA isolated from A2780 ovarian cancer cells was treated *in vitro* with different cisplatin and BBR 3464 concentrations for 2 hours at 37°C in the dark and then subjected to linear PCR amplification. Also in this case, drug concentrations were chosen on the basis of the results obtained in preliminary experiments carried out to ascertain the presence of single-hit kinetics in the system.

In the assay, a 16mer primer, previously labelled with ^{32}P at the 5' termini, was used to select, amplify and sequence a particular region of the cellular genome named alphoid DNA (α-DNA). The alphoid DNA is a satellite sequence that represents approximately 1% of the entire mammalian genome. Although this sequence does not play any significant role in controlling cellular metabolism, it can be considered an excellent target template for Taq stop assays in order to obtain very important information regarding the sequence specificity of DNA lesions generated by DNA damaging agents. In fact, in each mammalian cell, α-DNA is formed by about 100,000 copies of a 340 bp

tandemly repeated sequence named α-RI DNA, and it is separable in the 340 bp fragments by digestion with the EcoRI restriction endonuclease (Fig. 18). The sequence of such a DNA repeat is not perfectly homogeneous but contains random base substitutions (Fig. 18) which are 7% on average for each 340 bp repeat (Murray et al., 1992). However, the high copy number of this 340 bp monomer allows the straightforward determination of the sequence specificity of DNA damaging agents by Taq stop experiments. In fact, the use of thermal cycling to linearly amplify the products associated to the high copy number of template molecules dramatically increases the sensitivity of the system.

Data obtained from these Taq stop assays (Fig. 33) are in accord with those collected from experiments carried out using plasmid DNA molecules as the template. Results obtained from sequencing gel indicate the presence of six bands, all of them constituted by DNA molecules shorter than the full-length fragment and generated by premature blockage of the Taq polymerase activity, due to the presence of drug-induced adducts at 3'-GG-5', 3'-GA-5' and 3'-AG-5' dinucleotides. Results also indicate that the electrophoretic pattern obtained using naked genomic DNA exposed *in vitro* to the two drugs is more complex than that obtained using *in vitro*-treated plasmid DNA, probably as a consequence of the higher structural complexity of the DNA template. In these experiments, bands which did not show a clear dose-dependent increase of intensity (or in which the lack of such dose-dependent increase was not explainable by a lack of single-hit kinetics) as well as bands that in drug-treated samples exhibited an intensity comparable to that of untreated control sample were not considered as Taq stop sites.

Figure 33. Detection of adducts induced by BBR 3464 and cisplatin in naked genomic DNA after 2-hour *in vitro* drug exposure. The sites of DNA lesions are indicated as I-VI. The corresponding adduct sites on the DNA template (as determined from the consensus sequence) are underlined and possible base substitutions (Murray et al., 1992) are indicated in bold type.


Cisplatin (μM)
BBR 3464 (μM)
T A C G Ct 5 10 20 0.1 1 5
Full length
5'
3'
144
133
113
80
(VI)
3'-GTTACC-5'
GC GA
(V)
(IV)
3'-TCGGCGAA-5'
T G GG
(III)
(II)
(I)
3'-TGTGAGAA-5'
TC TC C

-Figure 33-

As already observed in Taq stop experiments carried out on plasmid DNA vector, also in this case it was possible to appreciate that the affinity of each drug for a specific dinucleotide seems to be influenced by neighbouring sequences. However, it is important to stress that the sequence heterogeneity among the copies of the 340 bp α-RI monomer has led to variability in the sequences which represent potential targets for adduct formation. As a consequence, the intensity of a band representing a specific stop site is probably the result of the sum of different adducts generated at the same position but on separated α-RI molecules. For example, the band corresponding to the site VI (Fig. 33) could be formed by molecules representing the products of premature Taq stop at different possible sites located in the same sequence, such as the 3'-GG-5' dinucleotide in α-RI monomers containing the 3'-GGTACC-5' sequence or 3'-AG-5' and 3'-GA-5' dinucleotides in α-RI monomers containing, in the same position, the 3'-GGTAGA-5' sequence.

Results obtained in these experiments indicate that the DNA damaging power of BBR 3464 is stronger than that of cisplatin. In fact, by considering the Taq stop site I, it is possible to observe that bands of comparable intensity were obtained with cisplatin concentrations (samples 5 and 10 μM) that are 10-50-fold greater than those of BBR 3464 (samples 0.1 and 1 μM) (Fig. 33).

The sequence specificity of adducts generated by cisplatin and BBR 3464 was then investigated under more physiological conditions by assessing the

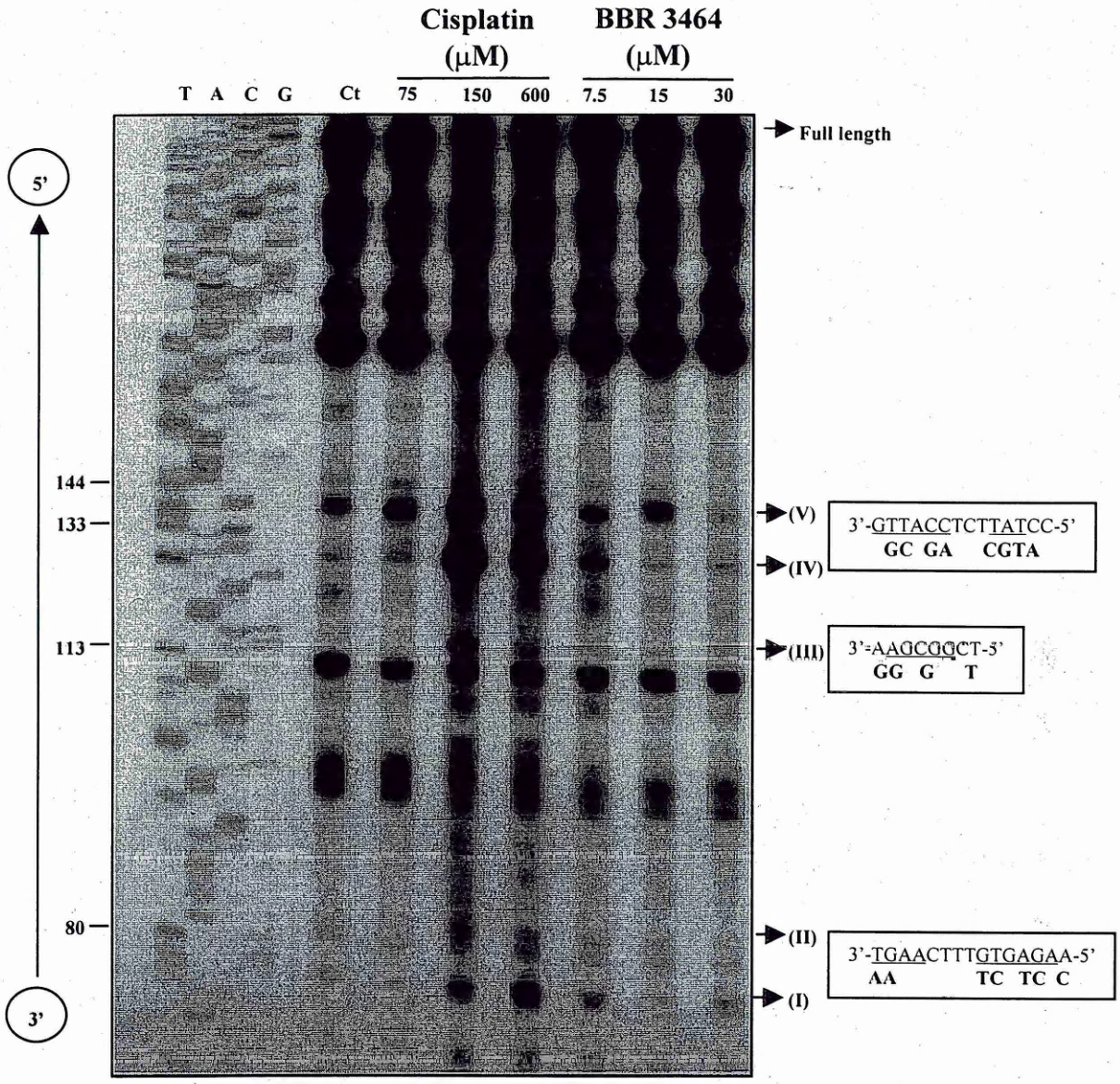
position of drug-induced Taq stop sites in the alphoid region of genomic DNA isolated after exposure of intact A2780 ovarian cancer cells to different concentrations of cisplatin or BBR 3464.

Drug concentrations were chosen on the basis of preliminary experiments carried out to verify whether single-hit kinetics were operative in the system.

Results, obtained from the sequencing gel shown in Figure 34, indicated that the two platinum-based compounds share almost the same sequence preference of adduct formation and that the DNA damaging power of BBR 3464 is greater than that of cisplatin. These findings are in accord with those obtained from Taq stop experiments carried out with naked plasmid pSP73 vector and genomic DNA treated *in vitro*. Specifically, five bands (I to V) corresponding to lesions generated at 3'-GG-5', 3'-GA-5' and 3'-AG-5' dinucleotides by cisplatin and BBR 3464 are clearly detectable. Only at site III there is a cisplatin-induced adduct which is not present in BBR 3464-treated samples. Also in this case, it is important to stress that each band representing a specific stop site is probably the result of the sum of different adducts generated at the same position but on separated α-RI molecules, as a consequence of the sequence heterogeneity among the different 340 bp monomers.

By comparing the results obtained from Taq stop assays carried out on naked genomic DNA treated *in vitro* to those on genomic DNA isolated from drug-treated living cells, it was possible to appreciate that some of the stop sites were detected in both situations. This finding would indicate that the sequences damaged by the two drugs are, at least in part, similar in the two different

Figure 34. Detection of adducts induced by BBR 3464 and cisplatin in genomic DNA isolated after a 2-hour exposure of intact A2780 cells to cisplatin and BBR 3464. The sites of DNA lesions are indicated as I-V. The corresponding adduct sites on the DNA template (as determined from the consensus sequence) are underlined and possible base substitutions (Murray et al., 1992) are indicated in bold type.


Cisplatin (μM)
BBR 3464 (μM)
T A C G
Ct
75
150
600
7.5
15
30
Full length
5'
3'
144
133
113
80
(V)
(IV)
(III)
(II)
(I)
3'-GTTACCTCTTATCC-5'
GC GA CGTA
3'-AAGCGCCT-5'
GG G T
3'-TGAACTTTGTGAGAA-5'
AA TC TC C

-Figure 34-

experimental environments, although 10 to 60-fold greater drug concentrations are needed to produce the same level of DNA damage under *in vivo* conditions.

4.7 Evaluation of the accumulation and repair of DNA damage induced by BBR 3464 and cisplatin at the single-gene level

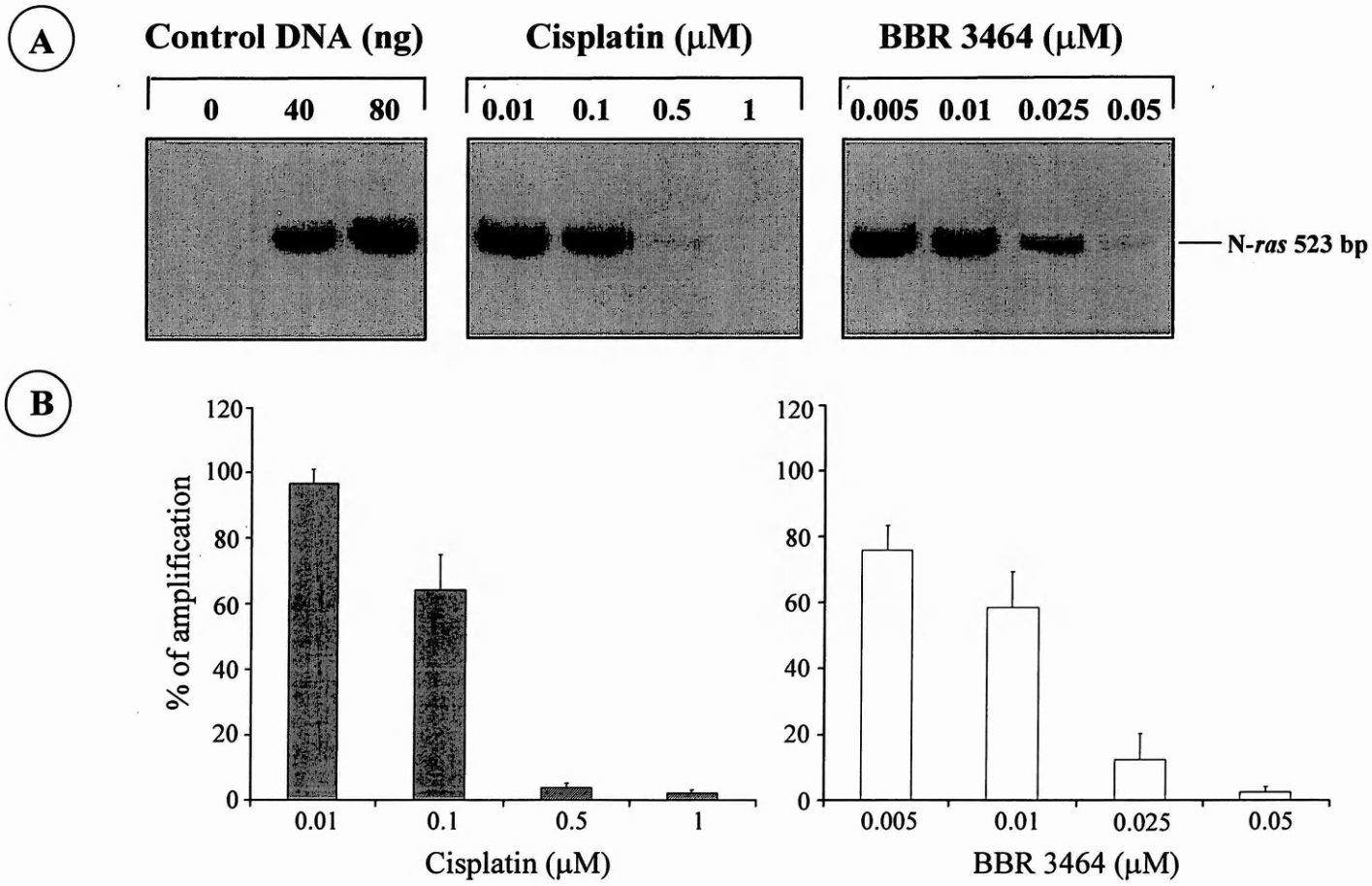
The kinetics of induction and removal of DNA lesions generated by cisplatin and BBR 3464 was measured at a single gene level using the quantitative PCR assay. As for the Taq stop assay, also in quantitative PCR the ability of drug-induced DNA adducts to halt Taq polymerase activity is exploited. However, differently from the Taq stop methodology, the quantitative PCR assay gives the possibility to accurately quantify the extent of DNA lesions generated by DNA damaging agents in a specific region of the genome. In particular, an opportunely selected pair of primers was used in PCR reactions to select and exponentially amplify a 523 bp sequence within the Intron I of the N-*ras* gene (Fig. 22). N-*ras* is an actively transcribed single copy gene which plays a relevant role in controlling cell cycle progression and which was already used as target substrate in similar studies (Bingham et al., 1996; Koberle et al., 1996 and 1997; O'Neill et al., 1999).

In order to obtain reliable quantitative information regarding the extent of the lesions generated on the cellular genome by DNA damaging agents, some fundamental experimental parameters were accurately set up. Specifically, the optimal amount of DNA substrate and the appropriate number of PCR cycles were defined in preliminary experiments.

To assess the sensitivity and reproducibility of the quantitative PCR technique, experiments were carried out using, as template, naked genomic DNA extracted from A2780 ovarian cancer cells and then exposed *in vitro* to various concentrations of cisplatin and BBR 3464 for 2 hours at 37°C in the dark. To ensure that amplification reactions were still in the exponential phase when stopped, the system was set up as follows. PCR samples containing a constant amount (300 ng) of damage-free DNA substrate were subjected to a different number of cycles and the amount of the radioactive product was quantified. After that the appropriate number of cycles was chosen, DNA substrate titration was performed in order to define the appropriate amount of DNA template to use in the successive experiments.

Figure 35A shows quantitative PCR results obtained by amplifying 80 ng of untreated and drug-treated naked genomic DNA for 26 cycles. Results indicated that the extent of signal amplification inhibition and, as a consequence, the level of lesions present in the N-*ras* gene, were dependent on drug concentration for both agents and confirmed the higher efficiency of BBR 3464 in inducing DNA lesions than cisplatin. In fact, the concentration of BBR 3464 required to induce a 50% amplification inhibition was 14 times lower than that of cisplatin (0.013 μM vs 0.183 μM) (Fig. 35B). In addition, it is important to stress that the intensity of the band corresponding to the untreated control sample containing 80 ng of DNA substrate is almost two-fold higher than that of control sample containing 40 ng of DNA template thus indicating that PCR was stopped when reactions still were in the exponential range.

Figure 35. (A) A representative quantitative PCR experiment on naked genomic DNA (N-*ras*) after 1-hour *in vitro* exposure to cisplatin and BBR 3464. Controls: 0, 40 and 80 ng of untreated DNA. **(B)** Quantification of PCR reactions for the N-*ras* gene. The ordinate shows the relative amplification expressed as percentage of control. Values represent the mean (± S.D.) of three independent experiments.

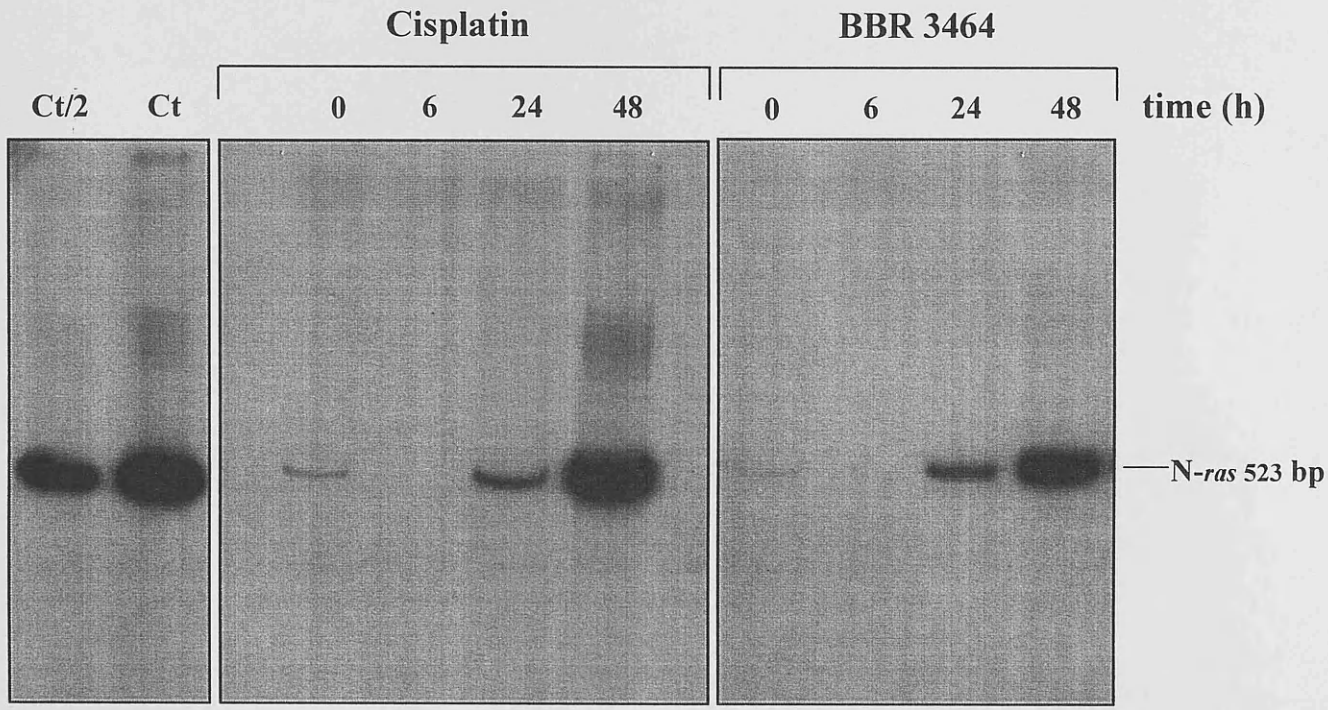

A
Control DNA (ng)
0
40
80
Cisplatin (μM)
0.01
0.1
0.5
1
BBR 3464 (μM)
0.005
0.01
0.025
0.05
N-ras 523 bp
B
% of amplification
120
100
80
60
40
20
0
0.01
0.1
0.5
1
Cisplatin (μM)
0.005
0.01
0.025
0.05
BBR 3464 (μM)

-Figure 35-

The effect of DNA damage present in the N-*ras* gene on PCR signal amplification was then evaluated in the four ovarian cancer cell lines immediately following a 5-hour exposure of intact cells to cisplatin or BBR 3464 and after an additional 6, 24 or 48 hour in drug-free medium. The experiments were carried out with IC_{50} of cisplatin in all cell lines. In the case of BBR 3464, 10 x IC_{50} was used, since in A2780, A2780cp8, and OAW42Mer cells the IC_{50} values of the trinuclear platinum compound were too low to produce a level of DNA damage reliably detectable by the PCR technique. Also in this case, preliminary experiments were performed to set up the experimental conditions of the system. Figure 36 shows a representative experiment of quantitative PCR in which 1,250 cell equivalents of genomic DNA isolated from untreated and drug-treated OAW42Mer living cells were used as template and subjected to thermal cycling for 26 cycles. Again, it is important to stress that the intensity of the band corresponding to the untreated control sample containing 1,250 cell equivalents of DNA substrate is almost two-fold higher than that of control sample containing 625 cell equivalents of DNA template thus indicating that PCRs were stopped when reactions still were in the exponential range.

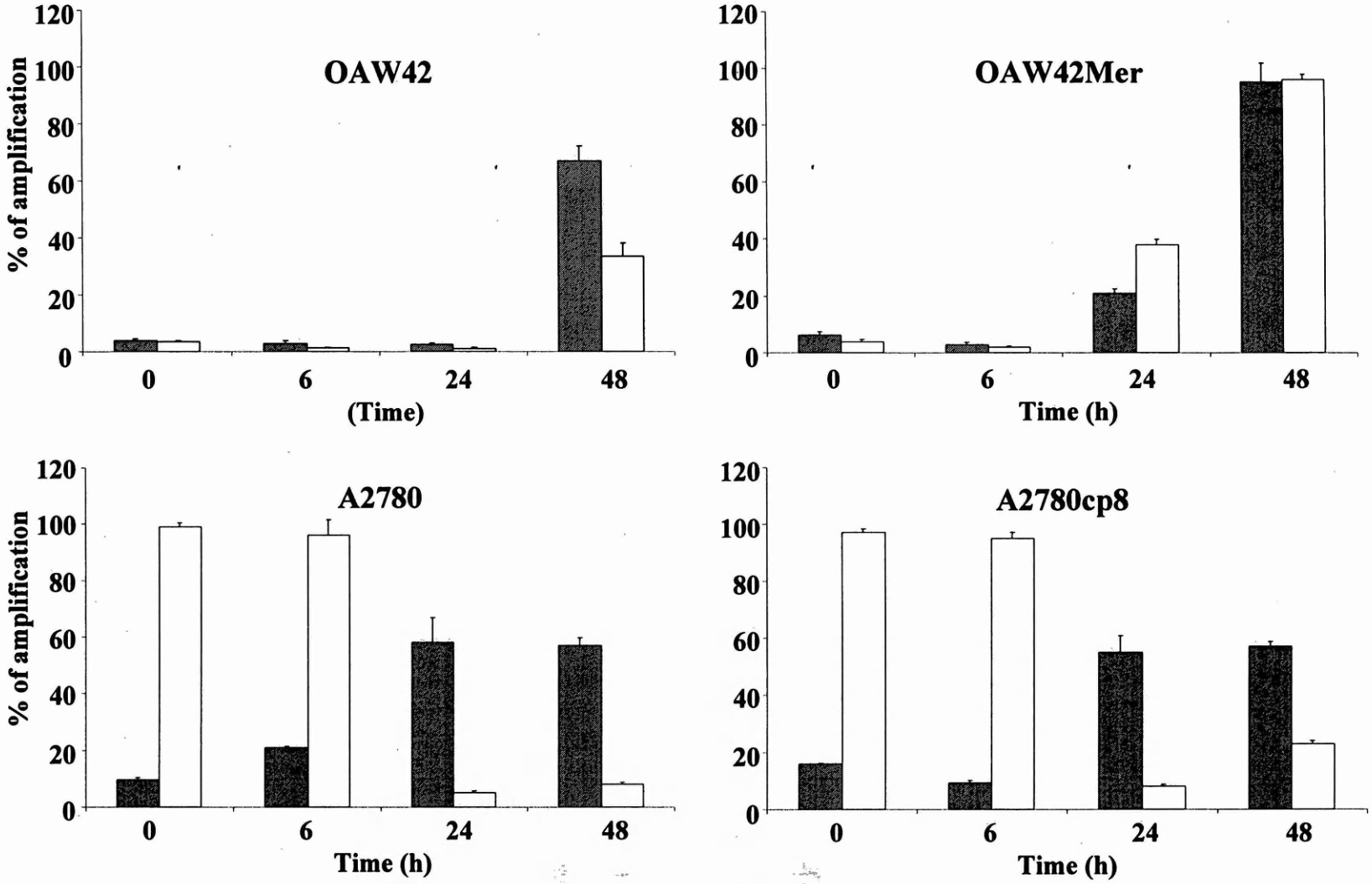
Data obtained from quantitative PCR in OAW42 cells (Fig. 37), evidenced a strong inhibition of signal amplification (3.8% of control) at the end of the 5-hour exposure to cisplatin (T_0). The level of inhibition remained almost the same 6 and 24 hours from the end of drug treatment (2.9% and 2.5% of control at T_6 and T_{24}, respectively) whereas at 48 hours the extent of amplification

Figure 36. A representative example of quantitative PCR analysis on genomic DNA (N-*ras* gene) after a 5-hour drug exposure of OAW42Mer cells to IC_{50} of cisplatin and 10 x IC_{50} of BBR 3464 and after an additional 6, 24 or 48 hours of incubation in drug-free medium. Controls: Ct/2 = 625 cell equivalents, Ct = 1,250 cell equivalents.



-Figure 36-

Figure 37. Quantification of PCR reactions for the N-*ras* gene following a 5-hour incubation to IC_{50} of cisplatin (▩) and 10 x IC_{50} of BBR 3464 (□) in OAW42, OAW42Mer, A2780 and A2780cp8 cell lines. The ordinate shows the relative amplification expressed as percentage of control. Values represent the mean (± S.D.) of three independent experiments.


OAW42
% of amplification
120
100
80
60
40
20
0
0
6
24
48
(Time)
OAW42Mer
Time (h)
A2780
A2780cp8

-Figure 37-

increased to 67% of control, thus indicating that a large number of adducts generated by cisplatin in the 523 bp region of N-*ras* gene Intron I were removed. Similar results were obtained after exposure of OAW42 cells to BBR 3464. Specifically, at the end of drug-treatment (T_0) amplification was dramatically inhibited (3.4% of control) and additional lesions were accumulated in the following 6 and 24 hours (T_6 and T_{24}: 1.2% and 1% of control, respectively). Differently from what was observed after cisplatin exposure, a large number of adducts was not removed after 48 hours of recovery in drug-free medium, so that the extent of amplification was significantly ($p<0.05$) lower and limited to 33.5% of control.

Immediately after OAW42Mer cells exposure to cisplatin (T_0), a strong inhibition of the signal amplification (6.2% of control) was observed and the accumulation of DNA lesions proceeded up to 6 hours (T_6) from drug withdrawal (2.8% of control). DNA damage was then partially repaired after 24 hours (T_{24}) of recovery in drug-free medium (21% of control), and only very few adducts were still present after 48 hour from the end of drug-exposure (95.8% of control) (Fig. 37). Similar kinetics of DNA adduct accumulation and removal was observed after treatment of OAW42Mer cells with BBR 3464. Specifically, a large number of DNA lesions was already present at the end of drug-treatment (T_0) and additional adducts were accumulated after 6 hours from drug withdrawal (T_6). At these time points, the signal amplification was limited to 3.9% and 2% of control, respectively. Removal of DNA lesions was already started after 24 hour (T_{24}: 38% of control) and almost completed after 48 hours (T_{48}: 96% of control). These data indicate that no significant

differences between cisplatin-sensitive and –resistant cell lines in the level of amplification inhibition or in extent of unrepaired damage are present. After drug exposure, some differences were observed only in the kinetics of DNA adducts processing after treatment with equitoxic concentrations of cisplatin. This was also true for BBR 3464.

As regards the second ovarian cancer cell line pair, in cisplatin-treated A2780 cells the maximum inhibition of signal amplification (9.6% of control) was found at the end of drug exposure (T_0) whereas a progressive removal of DNA lesions, with a consequent increase in the extent of amplification, was detected starting 6 hours after recovery in drug-free medium (T_6: 21% of control) (Fig. 37). Twenty-four hours from the end of treatment (T_{24}), the level of amplification reached 58.1% of control and no additional removal of DNA adducts was observed in the following 24 hours (T_{48}: 57% of control). In these cells a negligible inhibition of signal amplification was observed at the end (T_0: 99% of control) and after 6 hours from the end of BBR 3464 exposure (T_6: 96% of control), thus suggesting slow kinetics of DNA lesion induction. The highest level of amplification inhibition was detected 24 hours after drug withdrawal (T_{24}: 5% of control) and it was almost unmodified in the following 24 hours (T_{48}: 8% of control), thus indicating that DNA lesion removal did not occur.

After exposure of A2780cp8 cells to cisplatin, a kinetics of accumulation and removal of DNA lesion similar to those observed, with the same drug, in parental A2780 cells was detected, although in A2780cp8 cells the highest inhibition of signal amplification (which indicates the presence of the greatest

number of lesions) was detected after 6 hours from the end of drug treatment (T_0: 16% of control; T_6: 9.3 % of control) (Fig. 37). Also in these cells, slow kinetics of DNA adduct induction by BBR 3464 was recorded. The maximum inhibition of signal amplification was reached 24 hours after drug withdrawal (T_{24}: 8% of control) although in these cells, which showed a moderate degree of resistance to BBR 3464, a slight but significantly ($p<0.05$) higher increase in the level of amplification, with respect to what observed in A2780 cells, was appreciable 48 hours after the end of treatment (T_{48}: 23% of control), thus indicating a certain ability of these cells to partially remove DNA lesions generated by the novel trinuclear platinum complex.

4.8 Analysis of cell cycle perturbations induced by cisplatin and BBR 3464 in OAW42 and OAW42Mer cell lines

The analysis of the effects induced by BBR 3464 and cisplatin on cell cycle progression of OAW42 and OAW42Mer cells was carried out by flow cytometry with the aim being to determine whether cell cycle perturbations could be responsible, at least in part, for the different pattern of drug sensitivity observed in the two cell lines. Cells were exposed to the IC_{50} concentration of BBR 3464 (5.2 μM and 0.36 μM for OAW42 and OAW42Mer cells, respectively) and cisplatin (8.3 μM and 83 μM for OAW42 and OAW42Mer cells, respectively) for 1 hour, and their cell cycle distribution profiles were assessed at different intervals, (24, 48 and 72 hours) after drug withdrawal (Tab. XII).

Table XII. Cell cycle perturbation induced by cisplatin and BBR 3464. Data represent mean values ± SD of three independent experiments.

[1] Calculated from the end of a 1-hour treatment.

[2] Specific IC_{50} concentration.

[3] $P < 0.05$, Student's t-test, compared to controls.

	Time[1]								
	24 h			48 h			72 h		
	$G_{0/1}$	S	G_2M	$G_{0/1}$	S	G_2M	$G_{0/1}$	S	G_2M
<u>OAW42 cells</u>									
Control	63±5	24±5	13±1	62±5	25±5	12±1	66±7	21±7	13±1
BBR 3464[2]	37±6	18±7	45±5[3]	44±8	11±1	45±8[3]	50±8	10±1	38±9[3]
Cisplatin[2]	33±2	52±8[3]	15±9	21±3	22±7	57±6[3]	45±3	9±2	46±6[3]
<u>OAW42Mer cells</u>									
Control	50±7	33±5	17±2	53±5	34±4	13±1	58±5	29±4	13±1
BBR 3464[2]	29±3	29±3	42±5[3]	40±3	22±2	38±1[3]	40±1	34±3	26±2
Cisplatin[2]	17±3	56±6[3]	27±5	35±4	29±1	36±3[3]	48±1	35±1	17±1

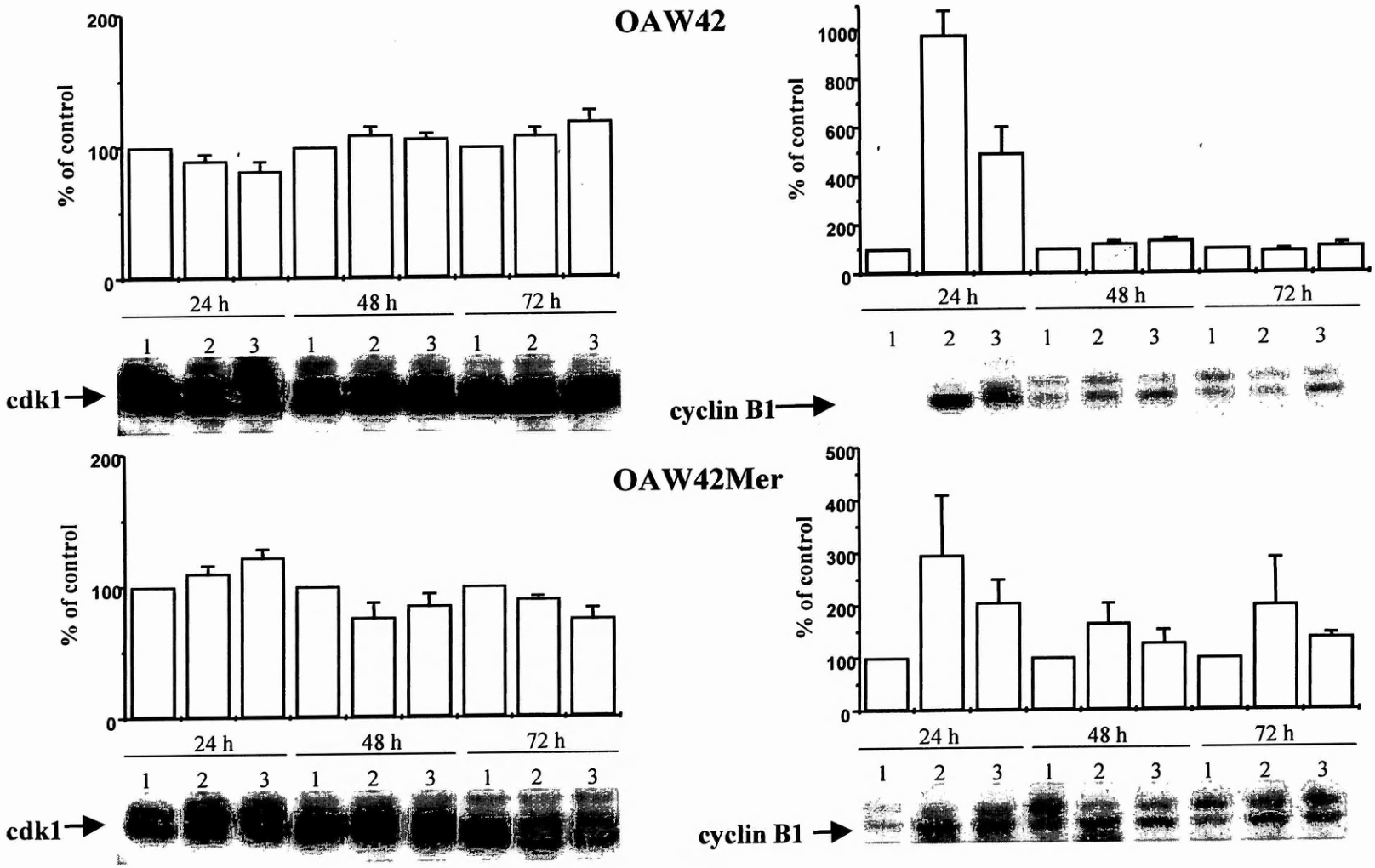
-Table XII-

In OAW42 cells, cisplatin induced a transient accumulation of cells in the S phase, appreciable 24 hours after the end of treatment, which was followed by a stable increase in the G_2M cell fraction, still present after 72 hours. As regards BBR 3464, after exposure to the trinuclear platinum complex OAW42 cells were stably blocked in the G_2M compartment until 72 hours. In OAW42Mer cells, exposure to cisplatin induced an accumulation of cells in the S phase at 24 hours followed by an increase in the G_2M cell fraction, which was almost completely resolved at 72 hours. In this cell line, BBR 3464 caused a persistent accumulation of cells in the G_2M phase, although less pronounced than that observed in OAW42 cells.

4.9 Evaluation of the effects induced by cisplatin and BBR 3464 on proteins involved in G_2 to M transition regulation

Since cisplatin and BBR 3464 mainly induced alterations in cell progression throughout the G_2M phase, the effect of drug treatment on the expression of cdk1 and cyclin B1 proteins, key regulators of the G_2 checkpoint, was determined (Fig. 38). To this purpose, OAW42 and OAW42Mer cells were exposed to the IC_{50} concentrations of cisplatin and BBR 3464 for 1 hour. Protein expression was then assessed by western blotting at 24, 48 and 72 hours after drug withdrawal and quantified by densitometric analysis of the autoradiographic films. Only differences in band intensities greater than 25% with respect to control were considered significant.

Figure 38. Effect of BBR 3464 and cisplatin on the expression of proteins involved in the control of G_2 checkpoint in OAW42 and OAW42Mer cells. Cells were incubated with solvent (control, lane 1) or with the IC_{50} concentration of BBR 3464 (lane 2) or cisplatin (lane 3) for 1 hour. At the end of treatment the cells were incubated for an additional 24, 48 and 72 hours in drug-free medium. Western blots, performed as described in Materials and Methods, were probed with antibodies for cdk1 and cyclin B1. The densitometric values of band intensities are indicated above the corresponding blots and represent the mean values ± SD of 3 independent experiments.


OAW42
% of control
200
100
0
24 h
48 h
72 h
1 2 3 1 2 3 1 2 3
cdk1
% of control
1000
800
600
400
200
0
24 h
48 h
72 h
1 2 3 1 2 3 1 2 3
cyclin B1
OAW42Mer
% of control
200
100
0
24 h
48 h
72 h
1 2 3 1 2 3 1 2 3
cdk1
% of control
500
400
300
200
100
0
24 h
48 h
72 h
1 2 3 1 2 3 1 2 3
cyclin B1

-Figure 38-

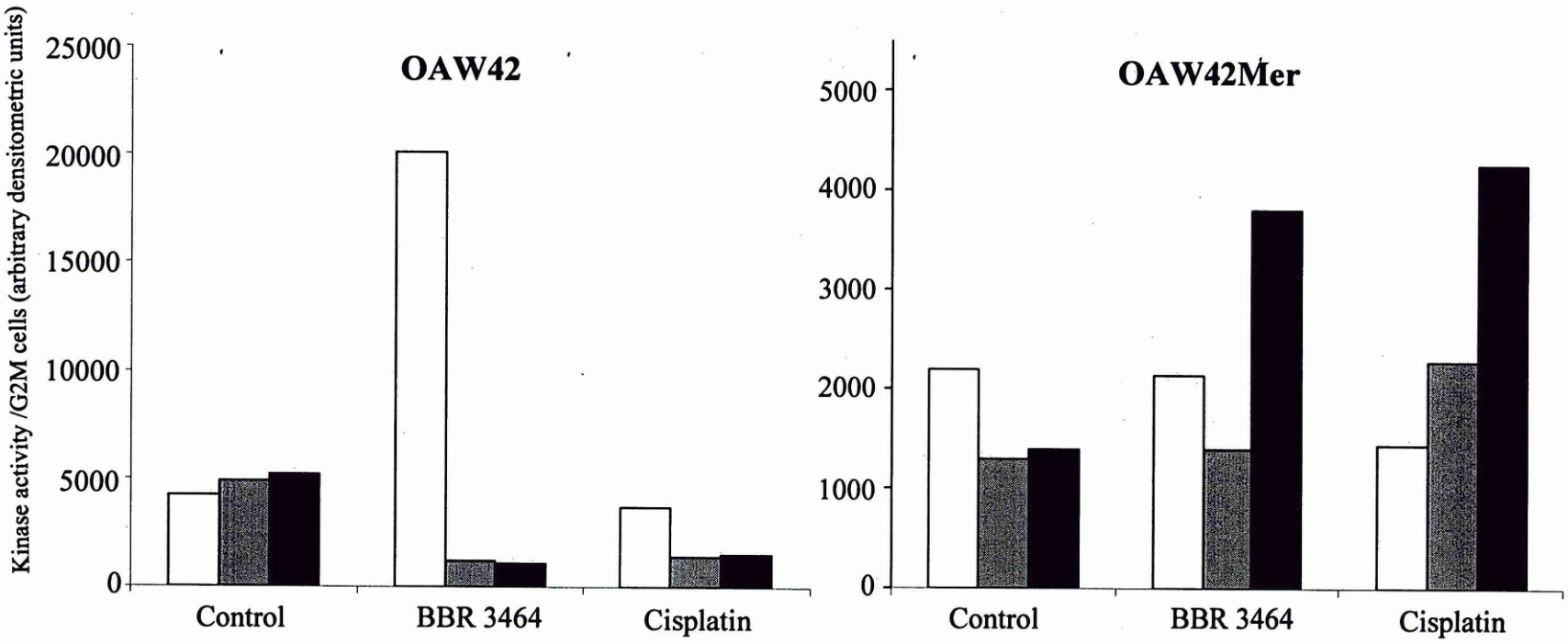
In OAW42 cells, BBR 3464 and cisplatin induced a marked increase in cyclin B1 expression that was highest 24 hour after treatment and still present, although to a lesser extent, at 72 hour (Fig. 38). No appreciable difference with respect to controls was found in cdk1 protein expression. Comparable findings were observed in OAW42Mer cells (Fig. 38)

The kinase activity of cyclin B1-associated cdk1 on the substrate histone H1 was also measured after drug treatment in both cell lines (Fig. 39). When the results were expressed in terms of kinase activity value divided by the number of G_2M cells (as detected by flow cytometry), we found that OAW42 cells accumulating in G_2M phase after cisplatin exposure showed a cdk1 catalytic activity consistently lower than that of control cells at all time points considered. In OAW42 cells exposed to BBR 3464, an unexpected and marked increase in cdk1 kinase activity was observed 24 hours after treatment, then the enzyme catalytic activity dropped to values lower than those observed in control cells. As regards the OAW42Mer cell line, cisplatin and BBR 3464-treated cells were characterised by levels of cdk1 kinase activity comparable to those of controls until 48 hours after treatment, whereas a marked increase in enzyme catalytic activity was observed at 72 hours in cells exposed to either drug (Fig. 39).

4.10 Evaluation of apoptosis induction by BBR 3464 and cisplatin

Activation of the programmed cell death pathway in cisplatin- and BBR 3464-treated OAW42 and OAW42Mer cells was determined by considering

Figure 39. Effect of BBR 3464 and cisplatin on cyclin B1/cdk1 kinase activity in OAW42 and OAW42Mer cells. Cells were incubated with solvent or with the IC_{50} concentration of BBR 3464 or cisplatin for 1 hour. At the end of the treatment, cells were incubated for an additional 24, 48 and 72 hours in drug-free medium. Immunoprecipitation and kinase assay were performed as described in Materials and Methods. Following autoradiography, reactions were quantified by densitometry; for each sample the results obtained at 24 (☐), 48 (▩) and 72 hours (■) were expressed in terms of kinase activity [arbitrary densitometric units (a.d.u.)] divided by the number of cells in the G_2M phase as assessed by flow cytometry.


Kinase activity /G2M cells (arbitrary densitometric units)
25000
20000
15000
10000
5000
0
OAW42
Control
BBR 3464
Cisplatin
5000
4000
3000
2000
1000
0
OAW42Mer
Control
BBR 3464
Cisplatin

-Figure 39-

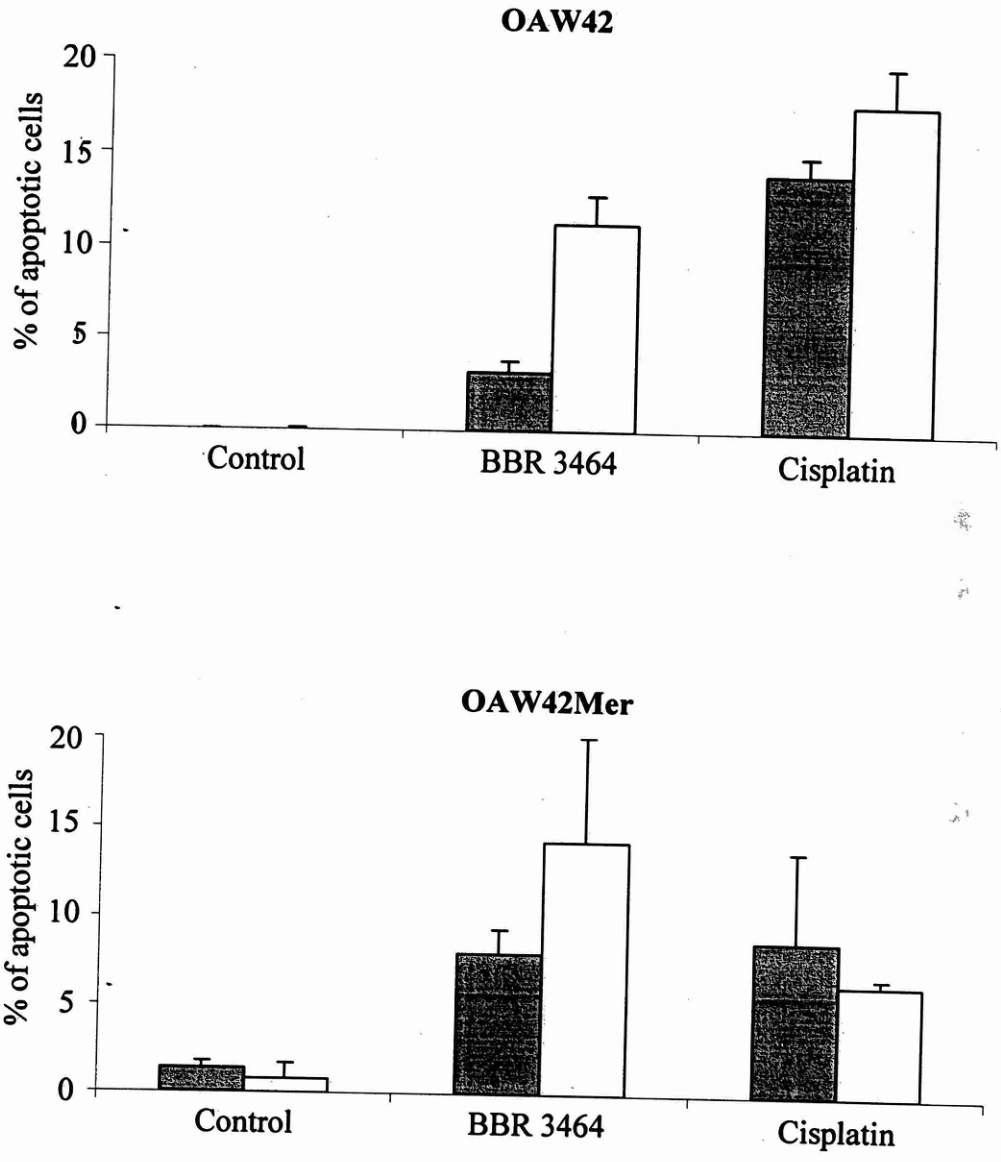
different end points. Cells were exposed to the IC_{50} concentrations of cisplatin and BBR 3464 for 1 hour, and the ability of the drugs to induce apoptosis was assessed 48 and 72 hours from the end of treatment.

The percentage of cells with an apoptotic nuclear morphology in drug-treated samples was initially determined by fluorescence microscopy after staining of cells with propidium iodide and calculated on the total cell population (Fig. 40). Spontaneous apoptosis was observed in a negligible fraction (<0.1%) of OAW42 control cells. Drug treatment induced a time-dependent increase in the percentage of OAW42 cells with an apoptotic morphology. This effect was slightly more pronounced for cisplatin (14-18%) than for BBR 3464 (3-12%). In untreated OAW42Mer cells spontaneous apoptosis was seen in about 1% of the overall cell population. This percentage increased after exposure of cells to cisplatin (7-9 %) or BBR 3464 (8-14 %).

Successively, the presence of DNA oligonucleosomal fragments in drug-treated samples was assessed by conventional DNA gel electrophoresis. Results of gel electrophoresis experiments carried out on DNA obtained from OAW42Mer cells showed the accumulation of oligonucleosome fragments 48 hours and 72 hours after treatment with cisplatin or BBR 3464 (Fig. 41). Similar results were also obtained in OAW42 cells.

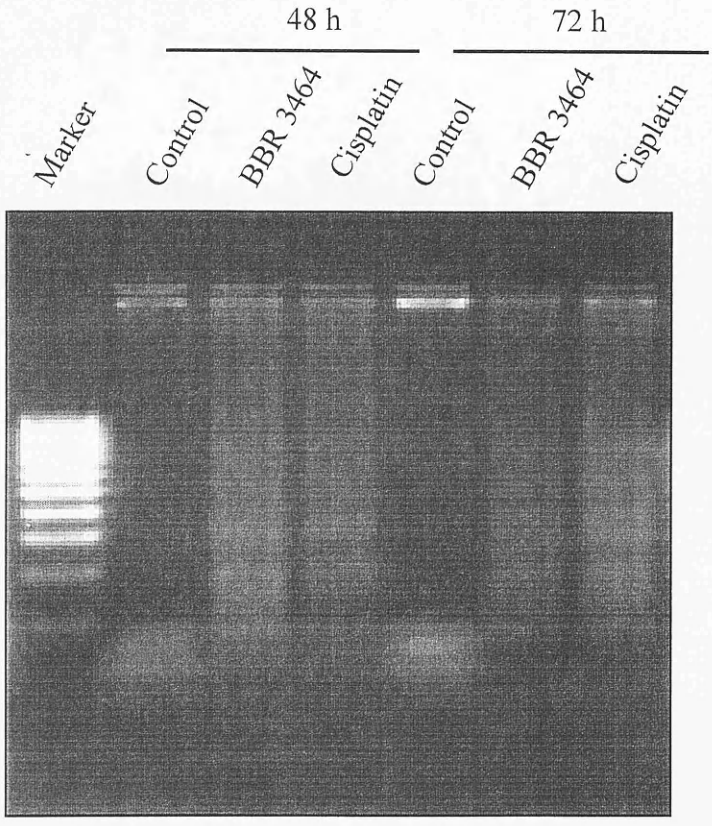
Degradation of nuclear lamin B, a specific substrate for terminal caspases, was also investigated by western blotting in drug-treated cells. Degradation of lamin B, as demonstrated by the appearance of a 45 KDa cleavage product and a concomitant decrease in the full-length protein level, was detected in OAW42

Figure 40. Induction of apoptosis by BBR 3464 and cisplatin in OAW42 and OAW42Mer cells. After a 1-hour treatment with solvent (control) or with the IC_{50} concentration of BBR 3464 or cisplatin, samples were incubated in drug-free medium and harvested after 48 (▩) and 72 hours (□). The cells were then stained with propidium iodide as described in Materials and Methods and the slides were examined by fluorescence microscopy. The percentage of apoptotic cells with respect to the total cell number was determined by scoring at least 200 cells in each sample. Data are means ± SD of three independent experiments.


OAW42
% of apoptotic cells
20
15
10
5
0
Control
BBR 3464
Cisplatin
OAW42Mer
% of apoptotic cells
20
15
10
5
0
Control
BBR 3464
Cisplatin

-Figure 40-

Figure 41. Electrophoretic pattern of DNA extracted from OAW42Mer floating cells. After a 1-hour treatment with solvent (control) or with the IC_{50} concentration of BBR 3464 or cisplatin, cells were incubated in drug-free medium and harvested after 48 and 72 hours.

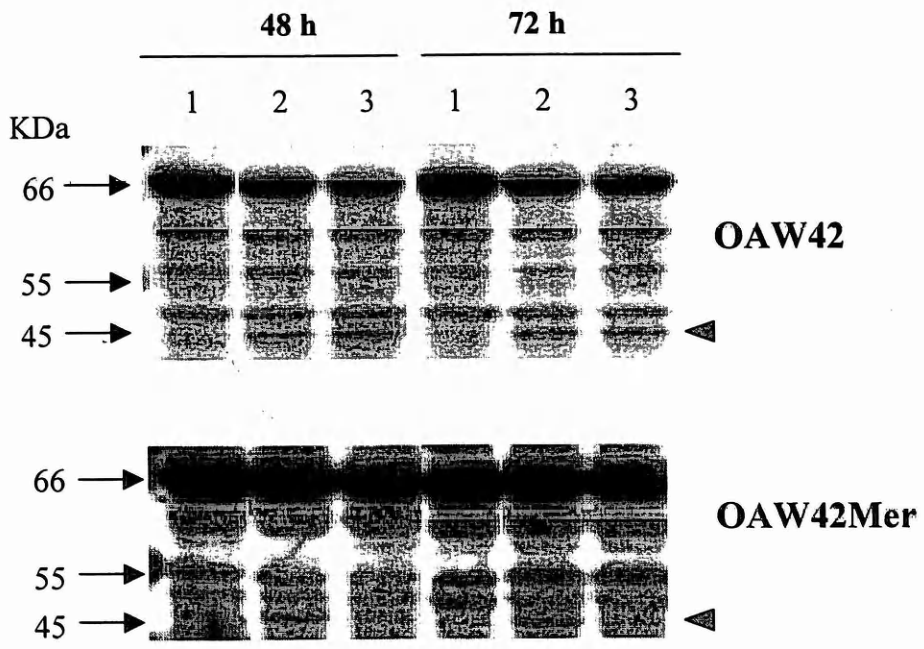

48 h
72 h
Marker
Control
BBR 3464
Cisplatin
Control
BBR 3464
Cisplatin

-Figure 41-

cells after exposure to cisplatin or BBR 3464. Conversely, in OAW42Mer cells lamin B cleavage was only detectable after BBR 3464 treatment (Fig. 42).

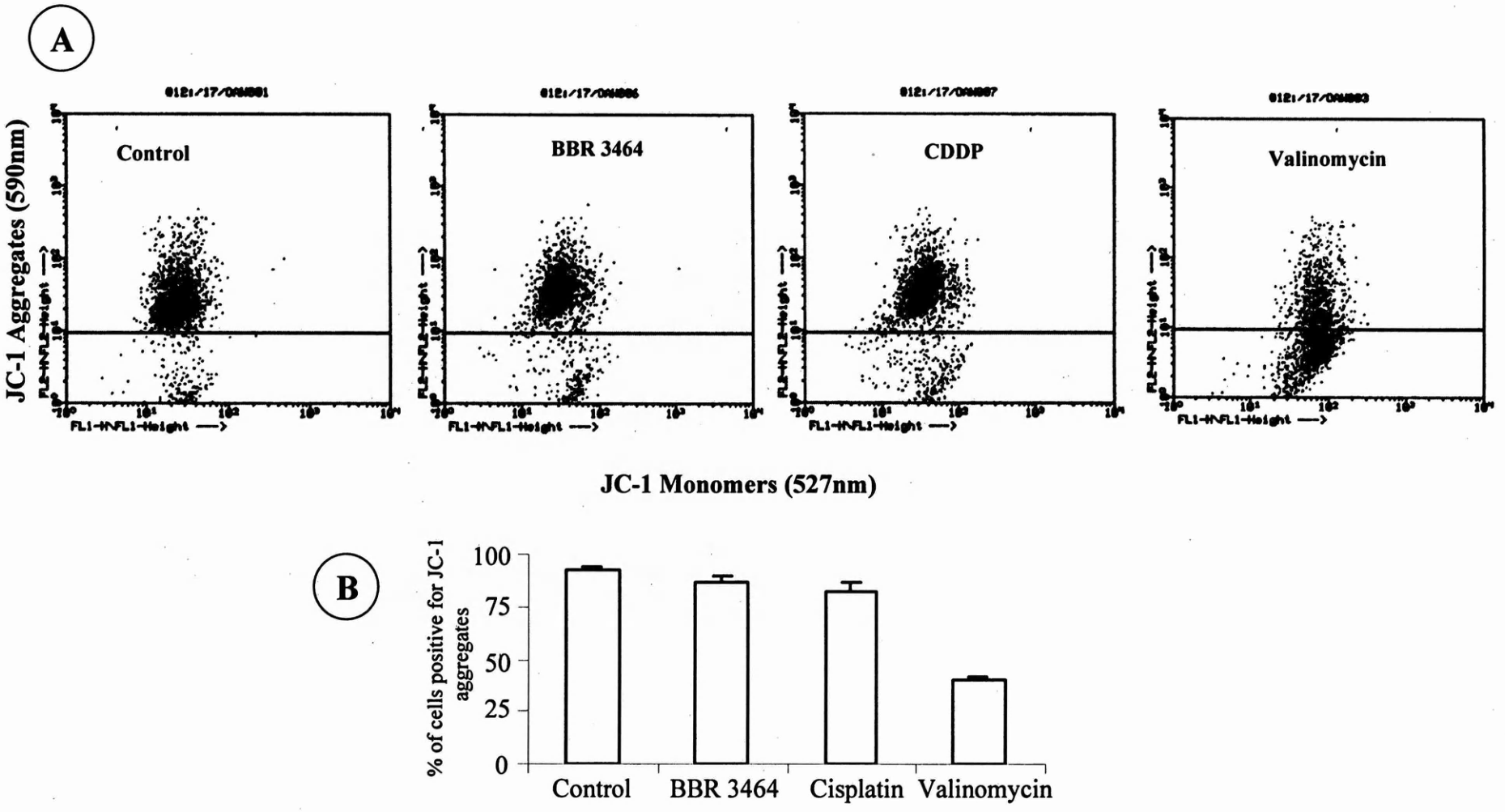
Since the disruption of mitochondrial membrane potential ($\Delta\psi_{mt}$) has been linked to the induction of apoptosis by different stimuli, we investigated whether the $\Delta\psi_{mt}$ was altered by cisplatin or BBR 3464 treatment in the two cell lines. For this purpose control and drug-treated cells were stained with the $\Delta\psi_{mt}$ -sensitive dye JC-1. At relatively high $\Delta\psi_{mt}$, the dye forms JC1-aggregates, which emit at 590 nm in the orange range of visible light. Conversely, in the absence of or at low $\Delta\psi_{mt}$, JC-1 exists as a monomer, remaining in the cell but emitting at 527 nm in the green range. Representative flow cytometric data, in which fluorescence at 590 nm is plotted against fluorescence at 527 nm, are shown in Figures 43 and 44. In untreated cells JC-1 exists predominantly in a highly aggregated form indicated by intense fluorescence emission at 590 nm. Conversely, in cells exposed to valinomycin, a K+ ionophore that uncouples oxidative phosphorylation, JC-1 was largely present in its monomeric state, thus indicating a low $\Delta\psi_{mt}$. As regards drug treatment, cisplatin and BBR 3464 induced negligible decreases in $\Delta\psi_{mt}$ of OAW42 cells (Fig. 43B). Conversely, in OAW42Mer cells cisplatin had almost no effect on $\Delta\psi_{mt}$ whereas after treatment of cells with BBR 3464 the $\Delta\psi_{mt}$ was markedly diminished, as demonstrated by the 29% reduction of cells positive for JC1-aggregates (Fig. 44B).

Figure 42. Degradation of lamin B induced by BBR 3464 or cisplatin in OAW42 and OAW42Mer cells. After a 1-hour treatment with the IC_{50} concentration of BBR 3464 or cisplatin, cells were incubated in drug-free medium and harvested after 48 and 72 hours. Western blotting was performed as described in Materials and Methods. The numbers on the left correspond to the migration position of protein markers. Lamin B-specific cleavage product bands are indicated on the right. Lanes: 1= untreated control sample; 2= BBR 3464-treated sample; 3= Cisplatin-treated sample. The blot of OAW42Mer cells has been overexposed to evidence the cleavage product.



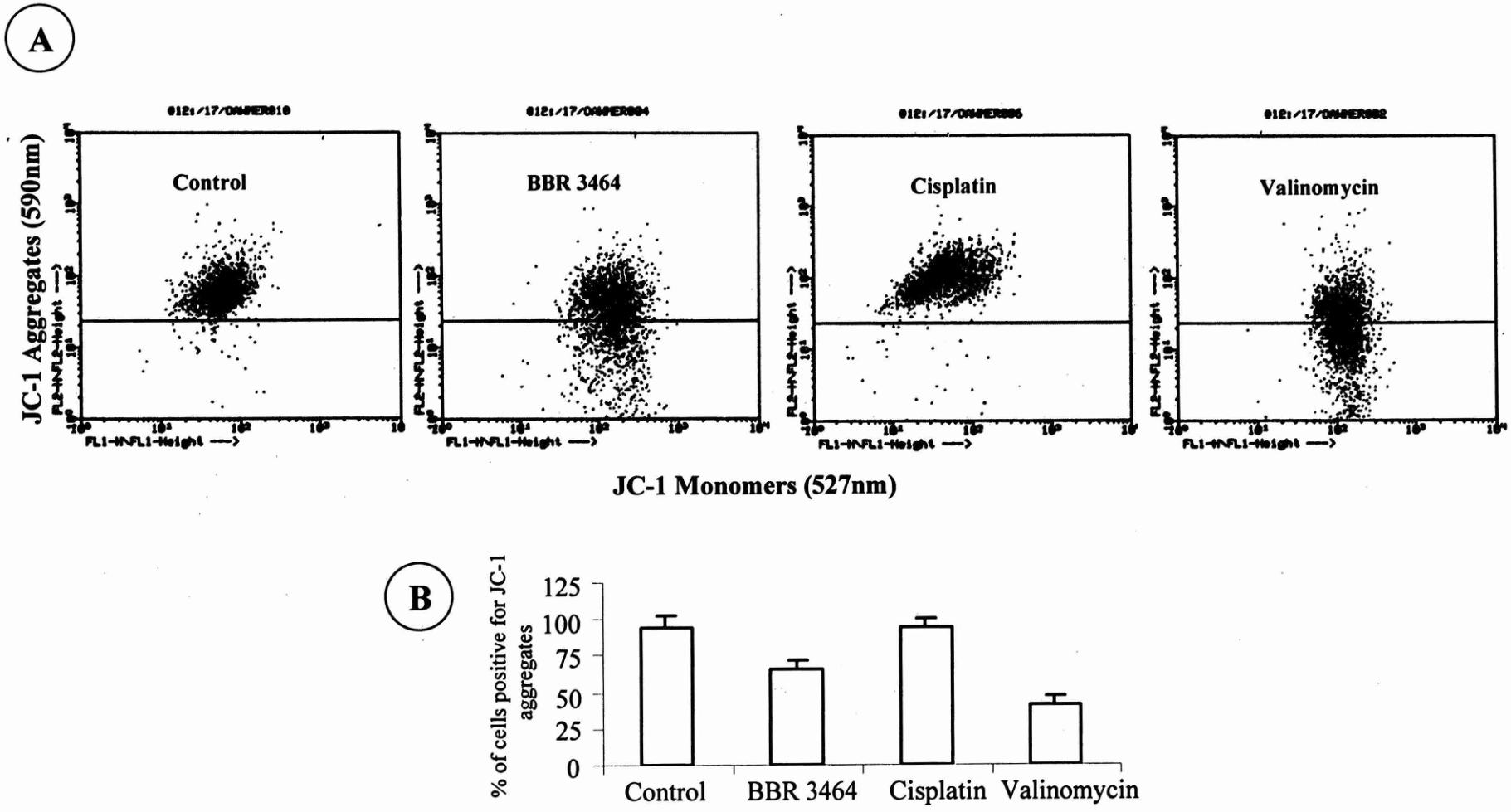
-Figure 42-

Figure 43.. Effect of BBR 3464 and cisplatin on the $\Delta\psi_{mt}$ in OAW42 cells. After a 1-hour treatment with IC_{50} concentration of BBR 3464 or cisplatin, cells were incubated in drug-free medium, stained with JC-1 and analysed by flow cytometry as described in Materials and Methods. At relatively high $\Delta\psi_{mt}$, JC-1 forms J-aggregates, which emit at 590 nm within the orange range of visible light. In contrast, in the absence of or at low $\Delta\psi_{mt,}$ JC-1 exists as a monomer, emitting at 527 nm in the green range. Cells treated for 30 min at 37°C prior to JC-1 addition with 5 μM valinomycin were used as positive control for $\Delta\psi_{mt}$ disruption. **(A)** Representative examples of the fluorescence pattern of cells incubated with the solvent (control) or with BBR 3464 or cisplatin. **(B)** Percentage of cells staining positive for J-aggregate formation (emitting at 590 nm) in untreated (control) and in BBR 3464 or cisplatin-treated cell populations. Data are means ± SD of three independent experiments.


A
Control
BBR 3464
CDDP
Valinomycin
JC-1 Aggregates (590nm)
JC-1 Monomers (527nm)
B
% of cells positive for JC-1 aggregates
100
75
50
25
0
Control
BBR 3464
Cisplatin
Valinomycin

-Figure 43-

Figure 44.. Effect of BBR 3464 and cisplatin on the $\Delta\psi_{mt}$ in OAW42Mer cells. **(A)** Representative examples of the fluorescence pattern of cells incubated with the solvent (control) or with BBR 3464 or cisplatin. **(B)** Percentage of cells staining positive for J-aggregate formation (emitting at 590 nm) in untreated (control) and BBR 3464 or cisplatin-treated cell populations. Data are means ± SD of three independent experiments.

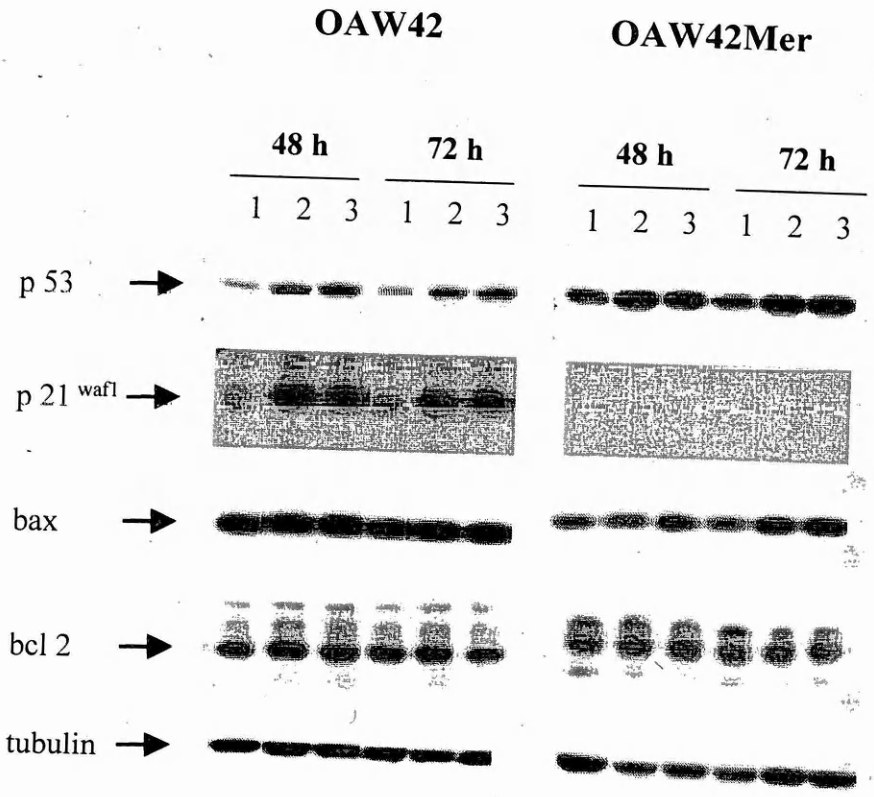

A
Control
BBR 3464
Cisplatin
Valinomycin
JC-1 Aggregates (590nm)
JC-1 Monomers (527nm)
B
% of cells positive for JC-1 aggregates
125
100
75
50
25
0
Control
BBR 3464
Cisplatin
Valinomycin

-Figure 44-

4.11 Evaluation of the effects induced by BBR 3464 and cisplatin on the expression of proteins involved in the control of apoptosis

Finally, the expression of proteins involved in the control of programmed cell death was assessed by western blotting analysis in OAW42 and OAW42Mer cells at different intervals (48 and 72 hours) after a 1-hour exposure to the IC_{50} concentration of cisplatin or BBR 3464 (Fig. 45). In OAW42 cells an increase in p53 protein expression and a consequent transactivation of $p21^{WAF1/CIP1}$ were observed after exposure to cisplatin or BBR 3464. In the OAW42Mer cell line p53 expression was also slightly increased after drug treatment, whereas the $p21^{WAF1/CIP1}$ signal remained undetectable. Moreover, drug treatment did not significantly modify bax expression in the two cell lines. As regards bcl-2, a slight reduction in protein levels was appreciable 72 hours after cisplatin exposure in the OAW42 cell line.

Figure 45. Effect of BBR 3464 and cisplatin on the expression of proteins involved in the control of apoptosis in OAW42 and OAW42Mer cells. Cells were incubated with solvent (control) or with the IC_{50} concentration of cisplatin or BBR 3464 for 1 hour. At the end of the treatment the cells were incubated for an additional 48 and 72 hours in drug-free medium. Western blots, performed as described in Materials and Methods, were probed with antibodies for p53, bcl-2, bax, and $p21^{waf1}$. Anti-tubulin α monoclonal antibody was used to ensure equal loading of protein on the gel. Lanes: 1= untreated control sample; 2= BBR 3464-treated sample; 3= Cisplatin-treated sample.


OAW42
OAW42Mer
48 h
72 h
48 h
72 h
1 2 3 1 2 3
1 2 3 1 2 3
p 53
p 21 wafl
bax
bcl 2
tubulin

-Figure 45-

DISCUSSION

(5)

Cisplatin is one of the most effective drugs for the treatment of a wide spectrum of human solid tumours. However, its clinical therapeutic efficacy is limited by the emergence of tumour cell subpopulations with intrinsic or treatment-induced resistance to the compound. Much effort has been dedicated to the development of new platinum-based molecules in order to overcome such resistance. Several mononuclear platinum compounds have been developed for this purpose (Kelland et al., 1992; Farrell, 1996), most of them showing a certain degree of cross-resistance to cisplatin as a consequence of the similarity in structure and molecular mechanism of action.

Multinuclear platinum compounds represent a different approach to circumvent cellular resistance to cisplatin in that they are endowed with a different DNA-binding profile compared to their mononuclear counterparts (Perego et al., 1999). BBR 3464 has been identified as the most active member of this class of compounds. The drug is more potent than cisplatin (Brabec et al., 1999) and is active in cisplatin-resistant experimental human tumour models (Pratesi et al., 1997; Manzotti et al.,2000). However, the cellular determinants responsible for BBR 3464 activity are largely unknown. Moreover, the relevance of factors contributing to cisplatin resistance, such as decreased drug accumulation (Loh et al., 1992; Mistry et al., 1992), increased detoxification (Fram et al., 1990; Mistry et al., 1991; Kasahara et al., 1991) and increased DNA repair and tolerance to DNA damage (Eastman and Schulte, 1988; Kelland et al., 1992b; Johnson et al., 1997; Hill et al., 1990; Behrens et al., 1987), in determining the cellular sensitivity profile to BBR 3464 needs to be established.

The ability of BBR 3464 to induce peculiar platinum-DNA adducts (such as 'long-distance' intra- and inter-strand cross-links (Farrell et al., 1995) which are not produced by conventional mononuclear platinum compounds suggests that BBR 3464 may escape, at least in part, the classical mechanisms of cisplatin resistance related to DNA damage recognition and repair (Yamada et al., 1997; Parker et al., 1991). Moreover, due to its ability to modify DNA in a way which is different from that of cisplatin, BBR 3464 could differently evoke pathways of cellular response to DNA damage such as triggering of the apoptotic pathway, as a function of the genetic background of the tumour model. In fact, it has recently been demonstrated that, unlike cisplatin which generally is less active against tumour models carrying a mutated p53 gene, BBR 3464 displays high activity in human tumour cell lines and xenografts characterised by mutant p53 (Pratesi et al., 1999), probably as a consequence of its ability to induce p53-independent programmed cell death.

In this study we focused on the cellular basis of the cytotoxic activity of BBR 3464 in human ovarian cancer cell lines sensitive to cisplatin and in their corresponding sublines selected *in vitro* for resistance to this agent. One of the cisplatin-resistant cell lines, OAW42Mer, showed a marked collateral sensitivity to BBR 3464 (being 14 times more sensitive to this agent than parental OAW42), whereas the second cisplatin-resistant cell line, A2780cp8, was characterised by a low degree of cross-resistance to BBR 3464.

It is well known that the status of DNA repair systems can be important in determining cellular response to alkylating agents and cisplatin (Aebi et al., 1996; Anthoney et al., 1996; Colella et al., 1999; Branch et al., 1993; Brown et

al., 1997; Drummond et al., 1996). Specifically, by removing DNA lesions, NER contributes to cellular resistance whereas MMR, by recognising the damage and generating apoptotic signals, contributes to the sensitivity of cells to the DNA damaging agent (Yamada et al., 1997). It has been reported that many cisplatin-resistant cell lines have a defective MMR system and present microsatellite instability (Fink et al., 1996; Aebi et al., 1996; Colella et al., 1999; Codegoni et al., 2000). Although mutations in several MMR genes have been found to be associated to the increased resistance observed in cancer cell lines to a variety of chemotherapeutic agents including cisplatin and carboplatin (Fink et al., 1998), several lines of evidence suggest a predominant role for hMLH1 in the acquisition of drug resistance in ovarian tumours. Specifically, an increased frequency of loss of hMLH1 expression has been observed in post-chemotherapy tumours compared to pre-chemotherapy samples (Brown et al., 1997). In addition, almost all of the cisplatin-resistant sublines derived from the A2780 ovarian cancer cell line showed a complete loss of hMLH1 protein expression. These resistant cells lack hMLH1 expression because of methylation of the hMLH1 gene promoter (Strathdee et al., 1999). To explain such a finding, it is possible to hypothesise that the A2780 cell population from which the resistant cell lines were derived contain a small subpopulation of cells with methylation of both alleles of hMLH1 gene, and that this MMR-deficient subpopulation is selected *in vitro* during exposure to cisplatin. This hypothesis is supported by findings that indicate the hMLH1 promoter as particularly susceptible to hypermethylation (Strathdee et al., 1999).

As for other cell lines (for example, the HCT-116 colon cancer cells), in which the lack of the expression of the hMLH1 protein was due to a hemizygous mutationin hMLH1 gene resulting in a truncated protein (Parson et al., 1993; Boyer et al., 1995), also in cisplatin-resistant A2780 cell line derivatives, re-expression of hMLH1 protein can be achieved by chromosome 3 transfer (Koi et al., 1994). Moreover, since in the cisplatin-resistant A2780 cell line derivatives the lack of hMLH1 expression is due to methylation-mediated gene silencing, there is the additional possibility to re-express hMLH1 by treatment with the demethylating agent 5-Azacytidine, a known inhibitor of the human DNA methyltransferase enzyme (Plumb et al., 2000). In preclinical studies carried out in human cancer cell lines and xenografts that are hMLH1 negative because of gene promoter hypermethylation, the treatment with non-toxic doses of 5-Azacytidine and DAC (2'-deoxy-5azacytidine) was found to be able to induce hMLH1 re-expression (Plumb et al., 2000). Moreover, it has been shown that DAC treatment sensitises drug-resistant human tumour xenografts to a number of clinically important cytotoxic drugs, thus raising the possibility that drug resistance mediated by methylation of hMLH1 could be overcome clinically.

Analysis of the MMR status in our cellular models evidenced that cisplatin-resistant cell lines OAW42Mer and A2780cp8 were characterised by deficiency in hMLH1 and hPMS2 proteins and showed microsatellite instability at a substantial number of loci. However, the lack of cross-resistance of OAW42Mer to BBR 3464 suggests that the cytotoxic activity of the trinuclear platinum compound is not dependent on the integrity of MMR. This

hypothesis is corroborated by previous findings of Perego et al. (1999), who showed that the absence of the hMLH1 protein in the colon cancer cell line HCT-116 or the hPMS2 protein in the osteogenic sarcoma cell line U2OS/Pt did not confer resistance to BBR 3464. The hypothesis is also in trend with data we obtained with the A2780d cell clone. In fact, A2780d cells, which were selected in culture from parental A2780 cells without drug exposure and are characterised by a defective MMR and microsatellite instability due to the lack of hMLH1 expression, show a moderate degree of resistance to cisplatin but maintain the same sensitivity to the trinuclear platinum compound when compared to the parental A2780 cell line.

A possible explanation for the selection of this MMR-defective cell clone is that the original population of parental A2780 cells was not homogeneous but contained a small population of cells in which lack of hMLH1 expression occurred by spontaneous gene-silencing through hypermethylation of the promoter of both alleles of hMLH1. It is possible that with respect to the mechanisms of selection proposed by Strathdee et al. (1999), in this specific case, the selective pressure for the MMR defective subpopulation selection could be represented by a long-time period of cell growth in culture flask. In fact, it is well known that cells defective for the MMR pathway are characterised not only by a reduced sensitivity to DNA damaging agents but also by a mutator phenotype which can confer to the cells some growth advantages.

As regards the NER pathway, an inverse correlation between intracellular levels of ERCC1 and cisplatin sensitivity has been reported. Specifically, low

levels of the protein appear to be responsible for cellular hypersensitivity to the drug whereas an increased expression can generate a modest degree of resistance to cisplatin (Yu et al., 1998; Damia et al., 1996). In our models we observed an increase in ERCC1 levels in the OAW42Mer cells whereas in the A2780cp8 cells ERCC1 levels were not different from those of the parental cell line. However, the high level of ERCC1 in both A2780 and A2780cp8 is comparable to that in OAW42Mer and 3-fold higher than the level in OAW42. In each cell line the overall NER activity might represent the sum of the activities of the different enzymes involved. Among these, ERCC1 seems to play an important role in this pathway since it is responsible for the incision step and acts upstream of the other enzymes such as DNA polymerases. In fact, it has been reported that in Chinese hamster ovarian cells overexpression of ERCC1 may enhance the sensitivity to alkylating agents because a greater number of DNA lesions are induced than the other enzymes involved in the pathway can repair (Bramson and Panasci, 1993). Moreover, Yu et al. (1998) reported that the ratio between a full-length active form of ERCC1 mRNA and a shorter inactive form derived from alternative splicing is associated with the cisplatin sensitivity/resistance profile. Overall, these findings could partially explain why among our cell lines those with a high level of ERCC1 (OAW42Mer, A2780 and A2780cp8) are more sensitive to BBR 3464 than the OAW42 cells, which express less protein. In fact, it is possible to speculate that in cells overexpressing ERCC1 the particular bulky DNA lesions generated by the trinuclear platinum compound are not completely processed by the NER pathway probably because they are able to induce ERCC1 to make more

incisions than the remaining NER enzymes are able to repair. To better evaluate the importance of cellular ERCC1 levels in determining cellular sensitivity to BBR 3464, additional experiments will be performed. For example, possible variations in cellular sensitivity to BBR 3464 will be determined after transfection of OAW42 cells with the human ERCC1 cDNA as well as with antisense constructs directed against the endogenous ERCC1 mRNA.

In an attempt to characterise the mechanisms responsible for the peculiar cytotoxicity profile of BBR 3464 observed in our cell models with respect to that of cisplatin, the cellular uptake of the drugs as well as the extent and type of interactions with DNA were determined. Results from atomic absorption spectrometry and mass spectroscopy experiments indicated that, while after exposure to cisplatin the intracellular accumulation of the drug and DNA platination levels are lower in the two cisplatin-resistant cell lines (OAW42Mer and A2780cp8) compared to the sensitive parental counterparts, after treatment with BBR 3464 consistent differences were not observed in the different cell lines independently of their sensitivity profile to this compound. It is important to stress that drug uptake experiments are only able to detect possible differences in the drug influx/efflux rates between resistant and sensitive cell lines. In fact, since the amount of platinum atoms present in the cells or bound to genomic DNA was measured immediately after a 1-hour drug exposure, the results are not influenced by a different activity of cellular detoxification systems or by different ability of cellular DNA repair pathways to remove DNA adducts generated by the two platinum-based compounds in the different

cell lines. Overall, our intracellular drug accumulation and DNA platination results did not explain the different sensitivity profile we obtained in the four experimental models. However, in accordance with results obtained in a previous study (Perego et al., 1999), our data indicated that intracellular accumulation and DNA binding are much higher for BBR 3464 than for cisplatin This finding is consistent with an increased affinity of BBR 3464 to DNA, due to the presence of two reactive platinum centres in the molecule and to the high positive charge (+4) that could contribute to drug interaction with DNA through electrostatic and hydrogen binding.

The sequence specificity of DNA damage caused by BBR 3464 and cisplatin was then mapped by a Taq stop assay in genomic DNA of ovarian cancer cells and the repetitive alphoid DNA was used as the target DNA. The sites of BBR 3464 adducts were almost identical to the sites of cisplatin adducts and were consistent with the motifs GG, AG or GA. A BBR 3464 sequence preference for d(GG) or d(G) sites previously was observed in the pSP73 plasmid (Brabec et al., 1999). Moreover, when a comparison was made between adduct formation in naked genomic DNA treated with drugs *in vitro* and in genomic DNA isolated after drug treatment of intact cells, a similar sequence-specific position of the damage was observed in both systems. However, a higher overall reactivity of BBR 3464 as compared to cisplatin was observed.

Gene-specific repair of lesions induced by BBR 3464 and cisplatin in the N-*ras* gene was then analysed in all cell lines by quantitative PCR. In the cell line pair OAW42 and OAW42Mer different lesion accumulation and removal kinetics were observed after drug treatment, whereas superimposable results

were obtained for BBR 3464 and cisplatin within each cell line. Moreover, the maximum number of lesions accumulated as well as the extent of DNA damage repaired after treatment with the two drugs was similar in the two cell lines and did not explain the different cytotoxicity profiles observed. In A2780 and A2780cp8 cells the extent and the kinetics of induction and repair of cisplatin lesions were similar. As regards BBR 3464, the results obtained in both cell lines showed an almost negligible accumulation of DNA lesions until 6 hours after drug withdrawal, thus suggesting a very slow conversion of monoadducts to bifunctional adducts after treatment of these cell lines with the trinuclear platinum complex. In fact, although theoretically Taq DNA polymerase is affected by each kind of adduct, the PCR stop assay is expected to reflect mainly bifunctional adducts and in particular intrastrand cross-links which represent the majority of drug adducts for cisplatin (Bubley et al., 1994; Murray et al., 1992) and probably for BBR 3464 as well (Brabec et al., 1999). Moreover, a lack of substantial conversion of BBR 3464 monoadducts to bifunctional adducts (in this case interstrand cross-links) up to 5 hours from the end of treatment was observed previously (Perego et al., 1999) in a pair of human osteosarcoma cell lines sensitive and with experimentally induced resistance to cisplatin. In A2780cp8 cells, which showed a moderate degree of resistance to BBR 3464, a higher degree of repaired DNA damage was observed after BBR 3464 exposure compared to A2780-sensitive cells.

Overall, these results suggest that the peculiar cytotoxicity profile of BBR 3464 probably is sustained by its ability to modify DNA in a way which is different from that of cisplatin. In fact, it has been demonstrated that antibodies

raised to cisplatin-adducted DNA did not recognise DNA modified by BBR 3464 (Brabec et al., 1999). However, since the different sensitivity to cisplatin and BBR 3464 observed in our cellular models might also be attributable to a different ability to activate other pathways of cellular response to DNA damage as a function of the genetic background of the tumour model, we decided to comparatively evaluate in our ovarian cancer cell lines the effects of cisplatin and BBR 3464 in terms of interference with cell cycle progression and induction of apoptosis.

As regards drug effects on cell cycle progression, a 1-hour treatment with the IC_{50} cisplatin concentration induced an early increase in the S-phase cell fraction that was followed by accumulation of cells in the G_2M compartment, whereas BBR 3464 (IC_{50}) caused a persistent arrest of cells in the G_2M phase. Similar, although less pronounced perturbations, were observed in OAW42Mer cells after drug exposure.

We investigated whether differences in cell cycle perturbation could be ascribed to differences in expression of proteins involved in control of the G_2 checkpoint. Since the major regulator of G_2 to M transition is the M phase-promoting factor (MPF), a complex constituted of the catalytic subunit cdk1 and of the regulatory subunit cyclin B1 (Lewin, 1990), we focused our attention on the expression level of the two proteins and also evaluated the effect of drug treatment on the cyclin B1-associated cdk1 kinase activity using histone H1 as a substrate. When kinase activity was expressed as a function of the number of cells in the G_2M compartment (i.e., the cells that, together with late S-phase cells, mainly account for this specific kinase activity), we found

that OAW42 cells accumulating in the G_2M phase after exposure to cisplatin or BBR 3464 generally showed a reduced ability to phosphorylate histone H1 with respect to untreated control cells. This observation is in agreement with previous findings obtained after cisplatin treatment in other experimental tumour models (Nishio et al., 1993). The only exception was a marked increase in cyclin B1-associated kinase activity found in cells accumulated in the G_2M phase 24 hours after treatment with BBR 3464. This increase could be sustained by the high level of cyclin B1 induced by treatment at this time point, which could allow increased formation of the cyclin B1-cdk1 active complex.

In OAW42Mer cells accumulating in G_2M after exposure to cisplatin or BBR 3464 the cyclin B1-associated kinase activities were almost superimposable to those of control cells until 48 hours after treatment. The inability of treated cells to escape the G_2 block could be explained by assuming that the active cyclin B1-cdk1 complexes are confined to the cytoplasmic compartment. In fact, it has previously been demonstrated that exclusion of cdk1 kinase activity from the nucleus may contribute to the cell cycle delay occurring after irradiation in HeLa-S1 cells (Kao et al., 1999). An increased kinase activity was observed at later intervals (72 hours) after cisplatin or BBR 3464 in correspondence with the resolution of G_2M blocks.

Since apoptosis is a major mode of cell death induced by several DNA damaging agents (Hickman, 1992), we evaluated the induction of apoptosis after exposure of OAW42 and OAW42Mer cells to cisplatin or BBR 3464. Fluorescence microscopy analysis indicated the presence of cells with an apoptotic nuclear morphology in both cell lines after treatment with either

drug. The occurrence of apoptosis was also confirmed by the presence of DNA fragmentation in both cell lines. Although the percentage of apoptotic cells was generally modest, there was a trend towards a correlation between cell sensitivity to a specific drug and apoptotic response. Specifically, the parental OAW42 cells (which are sensitive to cisplatin) showed a slightly higher percentage of apoptotic cells after treatment with cisplatin than they did following treatment with BBR 3464, whereas in OAW42Mer cells (which are sensitive to BBR 3464) an inverse pattern was observed.

At the molecular level we investigated the effects of drug treatment on the expression of proteins involved in the control of apoptosis such as p53 (Liebermann et al., 1995) and some of the major downstream genes controlled by p53 including $p21^{WAF1/CIP1}$, bax and bcl-2 (el-Deiry, 1998). In the OAW42 cell line exposure to cisplatin or BBR 3464 caused a marked increase in p53 expression and a consequent transactivation of $p21^{WAF1/CIP1}$. Moreover, no variation in the levels of bax and bcl-2 expression was observed after drug treatment. It is important to stress that both OAW42 and OAW42Mer cell lines are characterised by wild-type p53 gene as determined by DNA sequencing analysis. However, whereas in OAW42 cells, p53 was found to be able to transactivate downstream genes such as $p21^{WAF1/CIP1}$ as well as to induce a block of cells in G_1 phase after exposure to γ-radiation, these findings were not observed in OAW42Mer cell line. As previously reported by other investigators (Perego et al., 1997), it is possible that the lack of functional activity of the wild-type p53 protein observed in OAW42Mer cells is due to its stabilisation and compartmentalisation in the cytoplasm as a consequence of

the interaction with other proteins such as Mdm2. Such an hypothesis shows also the possibility to explain the high basal level of p53 detected by western blot analysis in OAW42Mer cells.

Overall, our findings indicate that the presence of a wild-type and functional p53 protein that allows cells to undergo p53-dependent apoptosis is an important determinant of tumour cell sensitivity to cisplatin, as already demonstrated in experimental and clinical studies (Righetti et al., 1996; Sugimoto et al., 1999; Jones et al., 1998). Conversely, the presence of an apparently non-functional p53 protein, as indicated by the lack of $p21^{WAF1/CIP1}$ induction in OAW42Mer cells, is not detrimental for the susceptibility of cells to BBR 3464. This observation is in agreement with previous findings of Pratesi et al. (1999), who demonstrated superior activity of the trinuclear platinum complex against p53-mutant human tumour xenografts as compared to those carrying the wild-type gene. These authors also showed that the transfer of functional p53 in a p53-null human osteosarcoma cell line resulted in a marked reduction of cellular sensitivity to BBR 3464. The efficacy of the trinuclear platinum complex in tumour cells with mutant or non-functional p53 protein suggests that the drug is able to induce a p53-independent response through the induction of specific DNA lesions. However, since in OAW42Mer cells the apoptotic response to BBR 3464 is quite modest, the efficacy of the trinuclear platinum complex could also be due to the induction of a persistent cytostatic effect resulting from the inability of cells to recognise and repair drug-induced DNA lesions.

There is increasing evidence for a major role of mitochondria in the apoptotic process. In fact, it has been demonstrated that apoptosis-inducing stimuli can trigger uncoupling of electron transport from ATP production, leading to a decrease in the $\Delta\psi_{mt}$ and a corresponding production of reactive oxygen species that are responsible for the oxidative degradation of mitochondrial components (Zamzami et al., 1995). The occurrence of mitochondrial changes during the apoptotic process induced by cisplatin in sensitive HeLa cells has recently been reported (Melendez-Zajgla et al., 1999). Moreover, a more general role of mitochondrial damage in determining the cytotoxic activity of this compound has previously been proposed by Zhang et al. (1994), who reported that the toxic activity of cisplatin in renal cortical slices was related to a rapid loss of mitochondrial protein-SH followed by a substantial decrease in Ca^{2+} uptake and a decline in mitochondrial membrane potential. We found that in OAW42 cells cisplatin and BBR 3464 did not appreciably affect the $\Delta\psi_{mt}$. Conversely, in OAW42Mer cells a marked decrease in $\Delta\psi_{mt}$ was only evident after exposure to BBR 3464, which indicates the impairment of mitochondrial membrane function as a possible determinant of the chemosensitivity to the trinuclear platinum complex observed in this cell line.

Disruption of mitochondrial membrane function also leads to the release of protease activators (Kluck et al., 1997). In particular, the release of cytochrome C from the mitochondria into the cytoplasm may play a central role in the activation of the executioner phase of apoptosis through its participation in the proteolytic activation of caspases (Matsuyama et al., 2000). To investigate

whether different executioners of apoptosis are involved in the pathways of programmed cell death induced by the two drugs in the same cellular models, we assessed the expression of lamin B, a well-known caspase substrate, in drug-treated cells. In fact, proteolysis of lamin A and B has been reported to occur during apoptosis induced by different stimuli (Kaufmann, 1989b; Shimizu et al., 1997; Oberhammer et al., 1994) in various cell lines. Cleavage of lamin B was clearly detected in OAW42 cells after treatment with BBR 3464 or cisplatin. Conversely, in OAW42Mer cells it was only observed after exposure to the trinuclear platinum complex. Since nuclear lamin disassembly and proteolysis involves hyperphosphorylation of lamins by lamin kinases and cleavage by caspases (Shimizu et al., 1998; Buendia et al., 1999), it may be hypothesised that in the different cellular settings the two drugs may differently activate the lamin pathway leading to apoptosis.

In conclusion, the results of our study would suggest BBR 3464 to be a promising drug for the treatment of human tumours resistant to cisplatin as a consequence of alterations in the DNA repair systems or inactivation of p53 function. Moreover, the evidence that BBR 3464 is able to induce apoptotic pathways which are different from those caused by cisplatin in the same tumour models suggests that multinuclear platinum complexes represent an entirely new class of DNA binding agents rather than simple cisplatin analogues.

BIBLIOGRAPHY

(6)

Aaltonen LA, Peltomaki P, Leach FS, Sistonen P, Pylkkanen L, Mecklin JP, Jarvinen H, Powell SM, Jen J, Hamilton SR, et al. Clues to the pathogenesis of familial colorectal cancer. Science. 1993 May 7;260(5109):812-6.

Abbott DW, Freeman ML, Holt JT. Double-strand break repair deficiency and radiation sensitivity in BRCA2 mutant cancer cells. J Natl Cancer Inst. 1998 Jul 1;90(13):978-85.

Abbott DW, Thompson ME, Robinson-Benion C, Tomlinson G, Jensen RA, Holt JT. BRCA1 expression restores radiation resistance in BRCA1-defective cancer cells through enhancement of transcription-coupled DNA repair. J Biol Chem. 1999 Jun 25;274(26):18808-12.

Aboussekhra A, Chanet R, Adjiri A, Fabre F. Semidominant suppressors of Srs2 helicase mutations of Saccharomyces cerevisiae map in the RAD51 gene, whose sequence predicts a protein with similarities to procaryotic RecA proteins. Mol Cell Biol. 1992 Jul;12(7):3224-34.

Acharya S, Wilson T, Gradia S, Kane MF, Guerrette S, Marsischky GT, Kolodner R, Fishel R. hMSH2 forms specific mispair-binding complexes with hMSH3 and hMSH6. Proc Natl Acad Sci U S A. 1996 Nov 26;93(24):13629-34.

Aebi S, Fink D, Gordon R, Kim HK, Zheng H, Fink JL, Howell SB. Resistance to cytotoxic drugs in DNA mismatch repair-deficient cells. Clin Cancer Res. 1997 Oct;3(10):1763-7.

Aebi S, Kurdi-Haidar B, Gordon R, Cenni B, Zheng H, Fink D, Christen RD, Boland CR, Koi M, Fishel R, Howell SB. Loss of DNA mismatch repair in acquired resistance to cisplatin. Cancer Res. 1996 Jul 1;56(13):3087-90.

Agarwal ML, Agarwal A, Taylor WR, Stark GR. p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. Proc Natl Acad Sci U S A. 1995 Aug 29;92(18):8493-7.

Akiyama S, Chen ZS, Sumizawa T, Furukawa T. Resistance to cisplatin. Anticancer Drug Des. 1999 Apr;14(2):143-51.

Akman S, Carr b, Leong L, Margolin K, Odujinrin O, Doroshows J. Phase I trial of menadiol sodium diphophate (Synkavite ®) (M) in advanced cancer. Proc ASCO 1988; 7:290.

Ali-Osman F, Berger MS, Rairkar A, Stein DE. Enhanced repair of a cisplatin-damaged reporter chloramphenicol-O-acetyltransferase gene and altered activities of DNA polymerases alpha and beta, and DNA ligase in cells of a human malignant glioma following in vivo cisplatin therapy. J Cell Biochem. 1994 Jan;54(1):11-9.

Andrews PA, Howell SB. Cellular pharmacology of cisplatin: perspectives on mechanisms of acquired resistance. Cancer Cells. 1990 Feb;2(2):35-43.

Andrews PA, In: Anticancer Drug Resistance: Advances in molecular and clinical Research. Goldstein LJ, Ozols RF (eds.) 1994; p. 127. Kluwer: Boston, MA USA.

Andrews PA, Mann SC, Huynh HH, Albright KD. Role of the Na+, K(+)-adenosine triphosphatase in the accumulation of cis-diamminedichloroplatinum(II) in human ovarian carcinoma cells. Cancer Res. 1991 Jul 15;51(14):3677-81.

Andrews PA, Murphy MP, Howell SB. Characterization of cisplatin-resistant COLO 316 human ovarian carcinoma cells. Eur J Cancer Clin Oncol. 1989 Apr;25(4):619-25.

Andrews PA, Murphy MP, Howell SB. Metallothionein-mediated cisplatin resistance in human ovarian carcinoma cells. Cancer Chemother Pharmacol. 1987;19(2):149-54.

Andrews PA, Schiefer MA, Murphy MP, Howell SB. Enhanced potentiation of cisplatin cytotoxicity in human ovarian carcinoma cells by prolonged glutathione depletion. Chem Biol Interact. 1988;65(1):51-8.

Anin MF, Leng M. Distortions induced in double-stranded oligonucleotides by the binding of cis- or trans-diammine-dichloroplatinum(II) to the d(GTG) sequence. Nucleic Acids Res. 1990 Aug 11;18(15):4395-400.

Anthoney DA, McIlwrath AJ, Gallagher WM, Edlin AR, Brown R. Microsatellite instability, apoptosis, and loss of p53 function in drug-resistant tumor cells. Cancer Res. 1996 Mar 15;56(6):1374-81.

Arrick BA, Nathan CF. Glutathione metabolism as a determinant of therapeutic efficacy: a review. Cancer Res. 1984 Oct;44(10):4224-32.

Baker SM, Bronner CE, Zhang L, Plug AW, Robatzek M, Warren G, Elliott EA, Yu J, Ashley T, Arnheim N, et al. Male mice defective in the DNA mismatch repair gene PMS2 exhibit abnormal chromosome synapsis in meiosis. Cell. 1995 Jul 28;82(2):309-19.

Baker SM, Plug AW, Prolla TA, Bronner CE, Harris AC, Yao X, Christie DM, Monell C, Arnheim N, Bradley A, Ashley T, Liskay RM. Involvement of

mouse Mlh1 in DNA mismatch repair and meiotic crossing over. Nat Genet. 1996 Jul;13(3):336-42.

Bakka A, Endresen L, Johnsen AB, Edminson PD, Rugstad HE. Resistance against cis-dichlorodiammineplatinum in cultured cells with a high content of metallothionein. Toxicol Appl Pharmacol. 1981 Nov;61(2):215-26.

Basile G, Aker M, Mortimer RK. Nucleotide sequence and transcriptional regulation of the yeast recombinational repair gene RAD51. Mol Cell Biol. 1992 Jul;12(7):3235-46.

Baumann P, West SC. Role of the human RAD51 protein in homologous recombination and double-stranded-break repair. Trends Biochem Sci. 1998 Jul;23(7):247-51.

Bedford P, Fichtinger-Schepman AM, Shellard SA, Walker MC, Masters JR, Hill BT. Differential repair of platinum-DNA adducts in human bladder and testicular tumor continuous cell lines. Cancer Res. 1988 Jun 1;48(11):3019-24.

Behrens BC, Hamilton TC, Masuda H, Grotzinger KR, Whang-Peng J, Louie KG, Knutsen T, McKoy WM, Young RC, Ozols RF. Characterization of a cis-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. Cancer Res. 1987 Jan 15;47(2):414-8.

Bellon SF, Coleman JH, Lippard SJ. DNA unwinding produced by site-specific intrastrand cross-links of the antitumor drug cis-diamminedichloroplatinum(II). Biochemistry. 1991 Aug 13;30(32):8026-35.

Boulton SJ, Jackson SP. Identification of a Saccharomyces cerevisiae Ku80 homologue: roles in DNA double strand break rejoining and in telomeric maintenance. Nucleic Acids Res. 1996 Dec 1;24(23):4639-48.

Boyer JC, Umar A, Risinger JI, Lipford JR, Kane M, Yin S, Barrett JC, Kolodner RD, Kunkel TA. Microsatellite instability, mismatch repair deficiency, and genetic defects in human cancer cell lines. Cancer Res. 1995 Dec 15;55(24):6063-70.

Brabec V, Kasparkova J, Vrana O, Novakova O, Cox JW, Qu Y, Farrell N. DNA modifications by a novel bifunctional trinuclear platinum phase I anticancer agent. Biochemistry. 1999 May 25;38(21):6781-90.

Brachman DG, Beckett M, Graves D, Haraf D, Vokes E, Weichselbaum RR. p53 mutation does not correlate with radiosensitivity in 24 head and neck cancer cell lines. Cancer Res. 1993 Aug 15;53(16):3667-9.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976 May 7;72:248-54.

Bramson J, Panasci LC. Effect of ERCC-1 overexpression on sensitivity of Chinese hamster ovary cells to DNA damaging agents. Cancer Res. 1993 Jul 15;53(14):3237-40.

Branch P, Aquilina G, Bignami M, Karran P. Defective mismatch binding and a mutator phenotype in cells tolerant to DNA damage. Nature. 1993 Apr 15;362(6421):652-4.

Britten RA, Warenius HM, White R, Browning PG, Green JA. Melphalan resistant human ovarian tumour cells are cross-resistant to photons, but not to high LET neutrons. Radiother Oncol. 1990 Aug;18(4):357-63.

Broggini M, Coley HM, Mongelli N, Pesenti E, Wyatt MD, Hartley JA, D'Incalci M. DNA sequence-specific adenine alkylation by the novel antitumor drug tallimustine (FCE 24517), a benzoyl nitrogen mustard derivative of distamycin. Nucleic Acids Res. 1995 Jan 11;23(1):81-7.

Broggini M, Erba E, Ponti M, Ballinari D, Geroni C, Spreafico F, D'Incalci M. Selective DNA interaction of the novel distamycin derivative FCE 24517. Cancer Res. 1991 Jan 1;51(1):199-204.

Bronner CE, Baker SM, Morrison PT, Warren G, Smith LG, Lescoe MK, Kane M, Earabino C, Lipford J, Lindblom A, et al. Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer. Nature. 1994 Mar 17;368(6468):258-61.

Brown R, Hirst GL, Gallagher WM, McIlwrath AJ, Margison GP, van der Zee AG, Anthoney DA. hMLH1 expression and cellular responses of ovarian tumour cells to treatment with cytotoxic anticancer agents. Oncogene. 1997 Jul 3;15(1):45-52.

Brown R. Mechanisms of cisplatin resistance: a post-DNA-damage perspective. FORUM Trends Exp Clin Med. 1995; 5:263-274.

Brown SJ, Kellett PJ, Lippard SJ. Ixr1, a yeast protein that binds to platinated DNA and confers sensitivity to cisplatin. Science. 1993 Jul 30;261(5121):603-5.

Bruhn SL, Pil PM, Essigmann JM, Housman DE, Lippard SJ. Isolation and characterization of human cDNA clones encoding a high mobility group box protein that recognizes structural distortions to DNA caused by binding of the anticancer agent cisplatin. Proc Natl Acad Sci U S A. 1992 Mar 15;89(6):2307-11.

Bubley GJ, Teicher BA, Ogata GK, Sandoval LS, Kusumoto T. Differences in in vivo and in vitro sequence-specific sites of cisplatin-DNA adduct formation and detection of a dose-response relationship. Biochem Pharmacol. 1994 Jul 5;48(1):145-53.

Buendia B, Santa-Maria A, Courvalin JC. Caspase-dependent proteolysis of integral and peripheral proteins of nuclear membranes and nuclear pore complex proteins during apoptosis. J Cell Sci. 1999 Jun;112 (Pt 11):1743-53.

Bungo M, Fujiwara Y, Kasahara K, Nakagawa K, Ohe Y, Sasaki Y, Irino S, Saijo N. Decreased accumulation as a mechanism of resistance to cis-diamminedichloroplatinum(II) in human non-small cell lung cancer cell lines: relation to DNA damage and repair. Cancer Res. 1990 May 1;50(9):2549-53.

Buttitta F, Marchetti A, Gadducci A, Pellegrini S, Morganti M, Carnicelli V, Cosio S, Gagetti O, Genazzani AR, Bevilacqua G. p53 alterations are predictive of chemoresistance and aggressiveness in ovarian carcinomas: a molecular and immunohistochemical study. Br J Cancer. 1997;75(2):230-5.

Butz K, Shahabeddin L, Geisen C, Spitkovsky D, Ullmann A, Hoppe-Seyler F. Functional p53 protein in human papillomavirus-positive cancer cells. Oncogene. 1995 Mar 2;10(5):927-36.

Caelles C, Helmberg A, Karin M. p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. Nature. 1994 Jul 21;370(6486):220-3.

Calsou P, Frit P, Salles B. Repair synthesis by human cell extracts in cisplatin-damaged DNA is preferentially determined by minor adducts. Nucleic Acids Res. 1992 Dec 11;20(23):6363-8.

Calsou P, Salles B. Role of DNA repair in the mechanisms of cell resistance to alkylating agents and cisplatin. Cancer Chemother Pharmacol. 1993;32(2):85-9.

Calvert AH, Harland SJ, Newell DR, Siddik ZH, Harrap KR. Phase I studies with carboplatin at the Royal Marsden Hospital. Cancer Treat Rev. 1985 Sep;12 Suppl A:51-7.

Canman CE, Gilmer TM, Coutts SB, Kastan MB. Growth factor modulation of p53-mediated growth arrest versus apoptosis. Genes Dev. 1995 Mar 1;9(5):600-11.

Carethers JM, Hawn MT, Chauhan DP, Luce MC, Marra G, Koi M, Boland CR. Competency in mismatch repair prohibits clonal expansion of cancer cells treated with N-methyl-N'-nitro-N-nitrosoguanidine. J Clin Invest. 1996 Jul 1;98(1):199-206.

Castellot JJ, Miller MR, Lehtomaki DM, Pardee AB. Comparison of DNA replication and repair enzymology using permeabilized baby hamster kidney cells. J Biol Chem. 1979 Aug 10;254(15):6904-8.

Castilla LH, Couch FJ, Erdos MR, Hoskins KF, Calzone K, Garber JE, Boyd J, Lubin MB, Deshano ML, Brody LC, et al. Mutations in the BRCA1 gene in families with early-onset breast and ovarian cancer. Nat Genet. 1994 Dec;8(4):387-91.

Chaney S. The chemistry and biology of platinum complexes with the 1,2-diaminocycloxane carrier ligand. Int J Oncol. 1995; 6:1291-1305.

Chao CC, Huang SL, Huang HM, Lin-Chao S. Cross-resistance to UV radiation of a cisplatin-resistant human cell line: overexpression of cellular factors that recognize UV-modified DNA. Mol Cell Biol. 1991 Apr;11(4):2075-80 (a).

Chao CC, Huang SL, Lee LY, Lin-Chao S. Identification of inducible damage-recognition proteins that are overexpressed in HeLa cells resistant to cis-diamminedichloroplatinum (II). Biochem J. 1991 Aug 1;277 (Pt 3):875-8 (b).

Chapman MS, Verma IM. Transcriptional activation by BRCA1. Nature. 1996 Aug 22;382(6593):678-9.

Chen CY, Oliner JD, Zhan Q, Fornace AJ, Vogelstein B, Kastan MB. Interactions between p53 and MDM2 in a mammalian cell cycle checkpoint pathway. Proc Natl Acad Sci U S A. 1994 Mar 29;91(7):2684-8

Chen ZS, Mutoh M, Sumizawa T, Furukawa T, Haraguchi M, Tani A, Saijo N, Kondo T, Akiyama S. An active efflux system for heavy metals in cisplatin-

resistant human KB carcinoma cells. Exp Cell Res. 1998 May 1;240(2):312-20.

Chu G, Chang E. Cisplatin-resistant cells express increased levels of a factor that recognizes damaged DNA. Proc Natl Acad Sci U S A. 1990 May;87(9):3324-8.

Chu G. Cellular responses to cisplatin. The roles of DNA-binding proteins and DNA repair. J Biol Chem. 1994 Jan 14;269(2):787-90.

Chuman Y, Chen ZS, Sumizawa T, Furukawa T, Haraguchi M, Takebayashi Y, Niwa K, Yamada K, Aikou T, Akiyama S. Characterization of the ATP-dependent LTC4 transporter in cisplatin-resistant human KB cells. Biochem Biophys Res Commun. 1996 Sep 4;226(1):158-65.

Chung DC, Rustgi AK. DNA mismatch repair and cancer. Gastroenterology. 1995 Nov;109(5):1685-99.

Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML, Wyllie AH. Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature. 1993 Apr 29;362(6423):849-52.

Clugston CK, McLaughlin K, Kenny MK, Brown R. Binding of human single-stranded DNA binding protein to DNA damaged by the anticancer drug cis-diamminedichloroplatinum (II). Cancer Res. 1992 Nov 15;52(22):6375-9.

Codegoni AM, Bertoni F, Colella G, Caspani G, Grassi L, D'Incalci M, Broggini M. Microsatellite instability and frameshift mutations in genes involved in cell cycle progression or apoptosis in ovarian cancer. Oncol Res. 1999;11(7):297-301.

Cole RS, Levitan D, Sinden RR. Removal of psoralen interstrand cross-links from DNA of Escherichia coli: mechanism and genetic control. J Mol Biol. 1976 May 5;103(1):39-59.

Cole RS. Repair of DNA containing interstrand crosslinks in Escherichia coli: sequential excision and recombination. Proc Natl Acad Sci U S A. 1973 Apr;70(4):1064-8.

Cole SP, Sparks KE, Fraser K, Loe DW, Grant CE, Wilson GM, Deeley RG. Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. Cancer Res. 1994 Nov 15;54(22):5902-10.

Colella G, Bonfanti M, D'Incalci M, Broggini M. Characterization of a protein recognizing minor groove binders-damaged DNA. Nucleic Acids Res. 1996 Nov 1;24(21):4227-33.

Colella G, Marchini S, D'Incalci M, Brown R, Broggini M. Mismatch repair deficiency is associated with resistance to DNA minor groove alkylating agents. Br J Cancer. 1999 May;80(3-4):338-43.

Crul M, Schellens JH, Beijnen JH, Maliepaard M. Cisplatin resistance and DNA repair. Cancer Treat Rev. 1997 Sep-Nov;23(5-6):341-66.

Cvitkovic E. Ongoing and unsaid on oxaliplatin: the hope. Br J Cancer. 1998 Jun;77 Suppl 4:8-11.

D'Atri S, Graziani G, Lacal PM, Tentori L, Bonmassar E, Pagani E, Jiricny J. Role of mismatch repair in apoptosis induced by methyltriazenes. Proc Am Assoc Cancer Res 1997; 38:1.

Dabholkar M, Bostick-Bruton F, Weber C, Bohr VA, Egwuagu C, Reed E. ERCC1 and ERCC2 expression in malignant tissues from ovarian cancer patients. J Natl Cancer Inst. 1992 Oct 7;84(19):1512-7.

Dabholkar M, Vionnet J, Bostick-Bruton F, Yu JJ, Reed E. Messenger RNA levels of XPAC and ERCC1 in ovarian cancer tissue correlate with response to platinum-based chemotherapy. J Clin Invest. 1994 Aug;94(2):703-8.

Damia G, Imperatori L, Stefanini M, D'Incalci M. Sensitivity of CHO mutant cell lines with specific defects in nucleotide excision repair to different anti-cancer agents. Int J Cancer. 1996 Jun 11;66(6):779-83.

Dancey J, Le Chevalier T. Non-small cell lung cancer: an overview of current management. Eur J Cancer. 1997 Jan;33 Suppl 1:2-7.

de Wind N, Dekker M, Berns A, Radman M, te Riele H. Inactivation of the mouse Msh2 gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer. Cell. 1995 Jul 28;82(2):321-30.

Dervan PB. Design of sequence-specific DNA-binding molecules. Science. 1986 Apr 25;232(4749):464-71.

Dethlefsen LA, Biaglow JE, Peck VM, Ridinger DN. Toxic effects of extended glutathione depletion by buthionine sulfoximine on murine mammary carcinoma cells. Int J Radiat Oncol Biol Phys. 1986 Jul;12(7):1157-60.

Di Leo A, Bajetta E, Biganzoli L, Bohm S, Mariani L, Menard S, Pilotti S, Fabbiani M, Gebbia V, Oriana S, et al. An I.T.M.O. group study on second-line treatment in advanced epithelial ovarian cancer: an attempt to identify

clinical and biological factors determining prognosis. Eur J Cancer. 1995 Dec;31A(13-14):2248-54.

Dignam JD, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. 1983 Mar 11;11(5):1475-89.

Donahue BA, Augot M, Bellon SF, Treiber DK, Toney JH, Lippard SJ, Essigmann JM. Characterization of a DNA damage-recognition protein from mammalian cells that binds specifically to intrastrand d(GpG) and d(ApG) DNA adducts of the anticancer drug cisplatin. Biochemistry. 1990 Jun 19;29(24):5872-80.

Dooley TP, Weiland KL, In: Methods in Molecular Biology: Drug-DNA Interaction Protocols. Fox KR (ed) 1997.Vol. 90: pp. 117-126: Human Press Inc., Totowa, NU USA.

Dornish JM, Pettersen EO, Oftebro R. Modifying effect of cinnamaldehyde and cinnamaldehyde derivatives on cell inactivation and cellular uptake of cis-diamminedichloroplatinum(II) in human NHIK 3025 cells. Cancer Res. 1989 Jul 15;49(14):3917-21.

Dosanjh MK, Collins DW, Fan W, Lennon GG, Albala JS, Shen Z, Schild D. Isolation and characterization of RAD51C, a new human member of the RAD51 family of related genes. Nucleic Acids Res. 1998 Mar 1;26(5):1179-84.

Douglas KT. Mechanism of action of glutathione-dependent enzymes. Adv Enzymol Relat Areas Mol Biol. 1987;59:103-67.

Drapkin R, Reardon JT, Ansari A, Huang JC, Zawel L, Ahn K, Sancar A, Reinberg D. Dual role of TFIIH in DNA excision repair and in transcription by RNA polymerase II. Nature. 1994 Apr 21;368(6473):769-72.

Drew HR. Structural specificities of five commonly used DNA nucleases. J Mol Biol. 1984 Jul 15;176(4):535-57.

Drummond JT, Anthoney A, Brown R, Modrich P. Cisplatin and adriamycin resistance are associated with MutLalpha and mismatch repair deficiency in an ovarian tumor cell line. J Biol Chem. 1996 Aug 16;271(33):19645-8.

Drummond JT, Li GM, Longley MJ, Modrich P. Isolation of an hMSH2-p160 heterodimer that restores DNA mismatch repair to tumor cells. Science. 1995 Jun 30;268(5219):1909-12.

Duckett DR, Drummond JT, Murchie AI, Reardon JT, Sancar A, Lilley DM, Modrich P. Human MutSalpha recognizes damaged DNA base pairs containing O6-methylguanine, O4-methylthymine, or the cisplatin-d(GpG) adduct. Proc Natl Acad Sci U S A. 1996 Jun 25;93(13):6443-7.

Dulic V, Kaufmann WK, Wilson SJ, Tlsty TD, Lees E, Harper JW, Elledge SJ, Reed SI. p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. Cell. 1994 Mar 25;76(6):1013-23.

Eastman A, Schulte N. Enhanced DNA repair as a mechanism of resistance to cis-diamminedichloroplatinum(II). Biochemistry. 1988 Jun 28;27(13):4730-4.

Eastman A. Activation of programmed cell death by anticancer agents: cisplatin as a model system. Cancer Cells. 1990 Aug-Sep;2(8-9):275-80.

Eastman A. Cross-linking of glutathione to DNA by cancer chemotherapeutic platinum coordination complexes. Chem Biol Interact. 1987 Mar;61(3):241-8.

el-Deiry WS, Harper JW, O'Connor PM, Velculescu VE, Canman CE, Jackman J, Pietenpol JA, Burrell M, Hill DE, Wang Y, et al. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res. 1994 Mar 1;54(5):1169-74.

el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. WAF1, a potential mediator of p53 tumor suppression. Cell. 1993 Nov 19;75(4):817-25.

el-Deiry WS. Regulation of p53 downstream genes. Semin Cancer Biol. 1998;8(5):345-57.

Eliopoulos AG, Kerr DJ, Herod J, Hodgkins L, Krajewski S, Reed JC, Young LS. The control of apoptosis and drug resistance in ovarian cancer: influence of p53 and Bcl-2. Oncogene. 1995 Oct 5;11(7):1217-28.

Enoch T, Norbury C.Cellular responses to DNA damage: cell-cycle checkpoints, apoptosis and the roles of p53 and ATM. Trends Biochem Sci. 1995 Oct;20(10):426-30.

Evans DL, Dive C. Effects of cisplatin on the induction of apoptosis in proliferating hepatoma cells and nonproliferating immature thymocytes. Cancer Res. 1993 May 1;53(9):2133-9.

Fajac A, Da Silva J, Ahomadegbe JC, Rateau JG, Bernaudin JF, Riou G, Benard J. Cisplatin-induced apoptosis and p53 gene status in a cisplatin-

resistant human ovarian carcinoma cell line. Int J Cancer. 1996 Sep 27;68(1):67-74.

Fan S, el-Deiry WS, Bae I, Freeman J, Jondle D, Bhatia K, Fornace AJ, Magrath I, Kohn KW, O'Connor PM. p53 gene mutations are associated with decreased sensitivity of human lymphoma cells to DNA damaging agents. Cancer Res. 1994 Nov 15;54(22):5824-30.

Fan S, Smith ML, Rivet DJ, Duba D, Zhan Q, Kohn KW, Fornace AJ, O'Connor PM. Disruption of p53 function sensitizes breast cancer MCF-7 cells to cisplatin and pentoxifylline. Cancer Res. 1995 Apr 15;55(8):1649-54.

Farrell N, Appleton TG, Qu Y, Roberts JD, Fontes AP, Skov KA, Wu P, Zou Y. Effects of geometric isomerism and ligand substitution in bifunctional dinuclear platinum complexes on binding properties and conformational changes in DNA. Biochemistry. 1995 Nov 28;34(47):145480-6.

Farrell N, In: Advances in DNA sequence specific agents. Hurley LH and Chayres JB (eds) 1996. pp. 187-216: JAI Press Inc., New Haven, CT USA.

Farrell N, Qu Y, Bierbach U, Valsecchi M, Menta E, In: 30 Years of Cisplatin-Chemistry and Biochemistry of a leading Anticancer Drug. Lippert B (ed) 1999. pp. 479-496: Verlag C.H., Basel Switzerland.

Farrell N, Qu Y, Feng L, Van Houten B. Comparison of chemical reactivity, cytotoxicity, interstrand cross-linking and DNA sequence specificity of bis(platinum) complexes containing monodentate or bidentate coordination spheres with their monomeric analogues. Biochemistry. 1990 Oct 16;29(41):9522-31.

Farrell N. Nonclassical platinum antitumor agents: perspectives for design and development of new drugs complementary to cisplatin. Cancer Invest. 1993;11(5):578-89.

Fink D, Aebi S, Howell SB. The role of DNA mismatch repair in drug resistance. Clin Cancer Res. 1998 Jan;4(1):1-6.

Fink D, Nebel S, Aebi S, Zheng H, Cenni B, Nehme A, Christen RD, Howell SB. The role of DNA mismatch repair in platinum drug resistance. Cancer Res. 1996 Nov 1;56(21):4881-6.

Fink D, Zheng H, Nebel S, Norris PS, Aebi S, Lin TP, Nehme A, Christen RD, Haas M, MacLeod CL, Howell SB. In vitro and in vivo resistance to cisplatin in cells that have lost DNA mismatch repair. Cancer Res. 1997 May 15;57(10):1841-5.

Fishel R, Ewel A, Lescoe MK. Purified human MSH2 protein binds to DNA containing mismatched nucleotides. Cancer Res. 1994 Nov 1;54(21):5539-42.

Fishel R, Lescoe MK, Rao MR, Copeland NG, Jenkins NA, Garber J, Kane M, Kolodner R. The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. Cell. 1993 Dec 3;75(5):1027-38.

Flores-Rozas H, Kelman Z, Dean FB, Pan ZQ, Harper JW, Elledge SJ, O'Donnell M, Hurwitz J. Cdk-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase delta holoenzyme. Proc Natl Acad Sci U S A. 1994 Aug 30;91(18):8655-9.

Fornace AJ, Zmudzka B, Hollander MC, Wilson SH. Induction of beta-polymerase mRNA by DNA-damaging agents in Chinese hamster ovary cells. Mol Cell Biol. 1989 Feb;9(2):851-3.

Fram RJ, Cusick PS, Wilson JM, Marinus MG. Mismatch repair of cis-diamminedichloroplatinum(II)-induced DNA damage. Mol Pharmacol. 1985 Jul;28(1):51-5.

Fram RJ, Woda BA, Wilson JM, Robichaud N. Characterization of acquired resistance to cis-diamminedichloroplatinum (II) in BE human colon carcinoma cells. Cancer Res. 1990 Jan 1;50(1):72-7.

Fraval HN, Roberts JJ. Excision repair of cis-diamminedichloroplatinum(II)-induced damage to DNA of Chinese hamster cells. Cancer Res. 1979 May;39(5):1793-7.

Friedberg EC. Relationships between DNA repair and transcription. Annu Rev Biochem. 1996;65:15-42.

Friedman HS, Johnson SP, Dong Q, Schold SC, Rasheed BK, Bigner SH, Ali-Osman F, Dolan E, Colvin OM, Houghton P, Germain G, Drummond JT, Keir S, Marcelli S, Bigner DD, Modrich P. Methylator resistance mediated by mismatch repair deficiency in a glioblastoma multiforme xenograft. Cancer Res. 1997 Jul 15;57(14):2933-6.

Friedman LS, Ostermeyer EA, Szabo CI, Dowd P, Lynch ED, Rowell SE, King MC. Confirmation of BRCA1 by analysis of germline mutations linked to breast and ovarian cancer in ten families. Nat Genet. 1994 Dec;8(4):399-404.

Friend S. p53: a glimpse at the puppet behind the shadow play. Science. 1994 Jul 15;265(5170):334-5.

Fritsche M, Haessler C, Brandner G. Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. Oncogene. 1993 Feb;8(2):307-18.

Fujii H, Shimada T. Isolation and characterization of cDNA clones derived from the divergently transcribed gene in the region upstream from the human dihydrofolate reductase gene. J Biol Chem. 1989 Jun 15;264(17):10057-64.

Fujii R, Mutoh M, Niwa K, Yamada K, Aikou T, Nakagawa M, Kuwano M, Akiyama S. Active efflux system for cisplatin in cisplatin-resistant human KB cells. Jpn J Cancer Res. 1994 Apr;85(4):426-33; (a).

Fujii R, Mutoh M, Sumizawa T, Chen ZS, Yoshimura A, Akiyama S. Adenosine triphosphate-dependent transport of leukotriene C4 by membrane vesicles prepared from cisplatin-resistant human epidermoid carcinoma tumor cells. J Natl Cancer Inst. 1994 Dec 7;86(23):1781-4; (b).

Fujiwara Y, Sugimoto Y, Kasahara K, Bungo M, Saijo N, Tew KD. Determinant of drug exposure in a cisplatin resistant human lung cancer cell line. Proc Am Assoc Cancer Res 1990; 31: 2187.

Fulop GM, Phillips RA. The scid mutation in mice causes a general defect in DNA repair. Nature. 1990 Oct 4;347(6292):479-82.

Futreal PA, Liu Q, Shattuck-Eidens D, Cochran C, Harshman K, Tavtigian S, Bennett LM, Haugen-Strano A, Swensen J, Miki Y, et al. BRCA1 mutations in primary breast and ovarian carcinomas. Science. 1994 Oct 7;266(5182):120-2.

Gately DP, Howell SB. Cellular accumulation of the anticancer agent cisplatin: a review. Br J Cancer. 1993 Jun;67(6):1171-6.

Giandomenico CM, Abrams MJ, Murrer BA et al., In: Platinum and Other Metal Coordination Compounds in Cancer Chemotherapy. Howell SB (ed) 1991. pp. 93-100: Plenum: New York NY USA.

Gibbons GR, Kaufmann WK, Chaney SG. Role of DNA replication in carrier-ligand-specific resistance to platinum compounds in L1210 cells. Carcinogenesis. 1991 Dec;12(12):2253-

Giuliani FC, Beggiolin G, Piazzoni L, Formento ML, Di Blasi P, arrell N, Kelland L, Caserini C, Perego p, Supino R, Zunino F. Pattern of cellular sensitivity to BBR 3464, a novel trinuclear platinum complex, in human tumor cell lines: lack of cross-resistance. Proc Am Assoc Cancer Res 1997; 38: 310.

Goddard PM, Valenti MR, Harrap KR. The role of murine tumour models and their acquired platinum-resistant counterparts in the evaluation of novel platinum antitumour agents: a cautionary note. Ann Oncol. 1991 Sep;2(8):535-40.

Gottesman MM, Pastan I. The multidrug transporter, a double-edged sword. J Biol Chem. 1988 Sep 5;263(25):12163-6.

Gowen LC, Avrutskaya AV, Latour AM, Koller BH, Leadon SA. BRCA1 required for transcription-coupled repair of oxidative DNA damage. Science. 1998 Aug 14;281(5379):1009-12.

Greenblatt MS, Bennett WP, Hollstein M, Harris CC. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res. 1994 Sep 15;54(18):4855-78.

Griffin S, Branch P, Xu YZ, Karran P. DNA mismatch binding and incision at modified guanine bases by extracts of mammalian cells: implications for tolerance to DNA methylation damage. Biochemistry. 1994 Apr 26;33(16):4787-93.

Griffith OW, Meister A. Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl homocysteine sulfoximine). J Biol Chem. 1979 Aug 25;254(16):7558-60.

Grimaldi KA and Hartley JA, In: Methods in Molecular Biology: Drug-DNA Interaction Protocols. Fox KR (ed) 1997.Vol. 90: pp. 157-180: Human Press Inc., Totowa, NU USA.

Grimaldi KA, Bingham JP, Souhami RL, Hartley JA. DNA damage by anticancer agents and its repair: mapping in cells at the subgene level with quantitative polymerase chain reaction. Anal Biochem. 1994 Oct;222(1):236-42 (a).

Grimaldi KA, McAdam SR, Souhami RL, Hartley JA. DNA damage by anti-cancer agents resolved at the nucleotide level of a single copy gene: evidence for a novel binding site for cisplatin in cells. Nucleic Acids Res. 1994 Jun 25;22(12):2311-7 (b).

Grosschedl R, Giese K, Pagel J. HMG domain proteins: architectural elements in the assembly of nucleoprotein structures. Trends Genet. 1994 Mar;10(3):94-100.

Haber JE. DNA recombination: the replication connection. Trends Biochem Sci. 1999 Jul;24(7):271-5.

Haldar S, Negrini M, Monne M, Sabbioni S, Croce CM. Down-regulation of bcl-2 by p53 in breast cancer cells. Cancer Res. 1994 Apr 15;54(8):2095-7.

Hamilton TC, Lai GM, Rothenberg ML, Fojo AT, Young RC, Ozols RF, In: Drug Resistance in Cancer Therapy. Mechanisms of resistance to cisplatin and alkylating agents. Ozols RF (ed) 1989. pp 151: Kluwer: Boston, MA USA.

Hamilton TC, Winker MA, Louie KG, Batist G, Behrens BC, Tsuruo T, Grotzinger KR, McKoy WM, Young RC, Ozols RF. Augmentation of adriamycin, melphalan, and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian carcinoma cell lines by buthionine sulfoximine mediated glutathione depletion. Biochem Pharmacol. 1985 Jul 15;34(14):2583-6.

Han HJ, Yanagisawa A, Kato Y, Park JG, Nakamura Y. Genetic instability in pancreatic cancer and poorly differentiated type of gastric cancer. Cancer Res. 1993 Nov 1;53(21):5087-9.

Hanawalt PC. DNA repair comes of age. Mutat Res. 1995 Mar;336(2):101-13.

Hanawalt PC. Transcription-coupled repair and human disease. Science. 1994 Dec 23;266(5193):1957-8.

Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell. 1993 Nov 19;75(4):805-16.

Harper JW, Elledge SJ, Keyomarsi K, Dynlacht B, Tsai LH, Zhang P, Dobrowolski S, Bai C, Connell-Crowley L, Swindell E, et al. Inhibition of cyclin-dependent kinases by p21. Mol Biol Cell. 1995 Apr;6(4):387-400.

Hartley JA, Berardini MD, Souhami RL. An agarose gel method for the determination of DNA interstrand crosslinking applicable to the measurement of the rate of total and "second-arm" crosslink reactions. Anal Biochem. 1991 Feb 15;193(1):131-4.

Hartley JA, Wyatt MD, In: Methods in Molecular Biology: Drug-DNA Interaction Protocols. Fox KR (ed) 1997.Vol. 90: pp. 147-156: Human Press Inc., Totowa, NU USA.

Hartwell L. Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. Cell. 1992 Nov 13;71(4):543-6.

Hartwell LH, Kastan MB. Cell cycle control and cancer. Science. 1994 Dec 16;266(5192):1821-8.

Hartwell LH, Szankasi P, Roberts CJ, Murray AW, Friend SH. Integrating genetic approaches into the discovery of anticancer drugs. Science. 1997 Nov 7;278(5340):1064-8.

Hartwell LH, Weinert TA. Checkpoints: controls that ensure the order of cell cycle events. Science. 1989 Nov 3;246(4930):629-34.

Havrilesky LJ, Elbendary A, Hurteau JA, Whitaker RS, Rodriguez GC, Berchuck A. Chemotherapy-induced apoptosis in epithelial ovarian cancers. Obstet Gynecol. 1995 Jun;85(6):1007-10.

Hawn MT, Umar A, Carethers JM, Marra G, Kunkel TA, Boland CR, Koi M. Evidence for a connection between the mismatch repair system and the G2 cell cycle checkpoint. Cancer Res. 1995 Sep 1;55(17):3721-5.

Hays SL, Firmenich AA, Berg P. Complex formation in yeast double-strand break repair: participation of Rad51, Rad52, Rad55, and Rad57 proteins. Proc Natl Acad Sci U S A. 1995 Jul 18;92(15):6925-9.

Hemminki A, Peltomaki P, Mecklin JP, Jarvinen H, Salovaara R, Nystrom-Lahti M, de la Chapelle A, Aaltonen LA. Loss of the wild type MLH1 gene is a feature of hereditary nonpolyposis colorectal cancer. Nat Genet. 1994 Dec;8(4):405-10.

Hendrickson EA, Qin XQ, Bump EA, Schatz DG, Oettinger M, Weaver DT. A link between double-strand break-related repair and V(D)J recombination: the scid mutation. Proc Natl Acad Sci U S A. 1991 May 15;88(10):4061-5.

Hess P, Aquilina G, Dogliotti E, Bignami M. Spontaneous mutations at aprt locus in a mammalian cell line defective in mismatch recognition. Somat Cell Mol Genet. 1994 Sep;20(5):409-21.

Hickman JA. Apoptosis induced by anticancer drugs. Cancer Metastasis Rev. 1992 Sep;11(2):121-39.

Hill BT, Shellard SA, Hosking LK, Fichtinger-Schepman AM, Bedford P. Enhanced DNA repair and tolerance of DNA damage associated with resistance to cis-diammine-dichloroplatinum (II) after in vitro exposure of a human teratoma cell line to fractionated X-irradiation. Int J Radiat Oncol Biol Phys. 1990 Jul;19(1):75-83.

Hoeijmakers JH, Bootsma D. DNA repair. Incisions for excision. Nature. 1994 Oct 20;371(6499):654-5

Hoffmann I, Clarke PR, Marcote MJ, Karsenti E, Draetta G. Phosphorylation and activation of human cdc25-C by cdc2--cyclin B and its involvement in the self-amplification of MPF at mitosis. EMBO J. 1993 Jan;12(1):53-63.

Hoffmann JS, Locker D, Villani G, Leng M. HMG1 protein inhibits the translesion synthesis of the major DNA cisplatin adduct by cell extracts. J Mol Biol. 1997 Jul 25;270(4):539-43.

Hoffmann JS, Pillaire MJ, Maga G, Podust V, Hubscher U, Villani G. DNA polymerase beta bypasses in vitro a single d(GpG)-cisplatin adduct placed on codon 13 of the HRAS gene. Proc Natl Acad Sci U S A. 1995 Jun 6;92(12):5356-60.

Hollander MC, Alamo I, Jackman J, Wang MG, McBride OW, Fornace AJ. Analysis of the mammalian gadd45 gene and its response to DNA damage. J Biol Chem. 1993 Nov 15;268(32):24385-93.

Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. Science. 1991 Jul 5;253(5015):49-53.

Holmgren A. Glutathione-dependent synthesis of deoxyribonucleotides. Purification and characterization of glutaredoxin from Escherichia coli. J Biol Chem. 1979 May 10;254(9):3664-71.

Horwich A, Mason M, Dearnaley DP. Use of carboplatin in germ cell tumors of the testis. Semin Oncol. 1992 Feb;19(1 Suppl 2):72-7.

Hospers GA, de Vries EG, Mulder NH. The formation and removal of cisplatin (CDDP) induced DNA adducts in a CDDP sensitive and resistant human small cell lung carcinoma (HSCLC) cell line. Br J Cancer. 1990 Jan;61(1):79-82 (a).

Hospers GA, Meijer C, de Leij L, Uges DR, Mulder NH, de Vries EG. A study of human small-cell lung carcinoma (hSCLC) cell lines with different sensitivities to detect relevant mechanisms of cisplatin (CDDP) resistance. Int J Cancer. 1990 Jul 15;46(1):138-44 (b).

Hospers GA, Mulder NH, de Jong B, de Ley L, Uges DR, Fichtinger-Schepman AM, Scheper RJ, de Vries EG. Characterization of a human small cell lung carcinoma cell line with acquired resistance to cis-diamminedichloroplatinum(II) in vitro. Cancer Res. 1988 Dec 1;48(23):6803-7.

Hromas RA, Andrews PA, Murphy MP, Burns CP. Glutathione depletion reverses cisplatin resistance in murine L1210 leukemia cells. Cancer Lett. 1987 Jan;34(1):9-13 (a).

Hromas RA, North JA, Burns CP. Decreased cisplatin uptake by resistant L1210 leukemia cells. Cancer Lett. 1987 Aug;36(2):197-201 (b).

Hsu LC, White RL. BRCA1 is associated with the centrosome during mitosis. Proc Natl Acad Sci U S A. 1998 Oct 27;95(22):12983-8.

Huang J, Papadopoulos N, McKinley AJ, Farrington SM, Curtis LJ, Wyllie AH, Zheng S, Willson JK, Markowitz SD, Morin P, Kinzler KW, Vogelstein B, Dunlop MG. APC mutations in colorectal tumors with mismatch repair deficiency. Proc Natl Acad Sci U S A. 1996 Aug 20;93(17):9049-54.

Huang JC, Zamble DB, Reardon JT, Lippard SJ, Sancar A. HMG-domain proteins specifically inhibit the repair of the major DNA adduct of the anticancer drug cisplatin by human excision nuclease. Proc Natl Acad Sci U S A. 1994 Oct 25;91(22):10394-8.

Huang LC, Clarkin KC, Wahl GM. Sensitivity and selectivity of the DNA damage sensor responsible for activating p53-dependent G1 arrest. Proc Natl Acad Sci U S A. 1996 May 14;93(10):4827-32.

Hughes EN, Engelsberg BN, Billings PC. Purification of nuclear proteins that bind to cisplatin-damaged DNA. Identity with high mobility group proteins 1 and 2. J Biol Chem. 1992 Jul 5;267(19):13520-7.

Hurley LH, Reynolds VL, Swenson DH, Petzold GL, Scahill TA. Reaction of the antitumor antibiotic CC-1065 with DNA: structure of a DNA adduct with DNA sequence specificity. Science. 1984 Nov 16;226(4676):843-4.

Husain A, He G, Venkatraman ES, Spriggs DR. BRCA1 up-regulation is associated with repair-mediated resistance to cis-diamminedichloroplatinum(II). Cancer Res. 1998 Mar 15;58(6):1120-3.

Inagaki K, Sawaki K. Reaction of (1R,2S,3S)-3-methylcyclohexanediamineplatinum(II) with DNA: isolation and characterization of the platinum-nucleotide adducts by means of HPLC and NMR spectroscopy. Chem Pharm Bull (Tokyo). 1995 Feb;43(2):183-8.

Irminger-Finger I, Siegel BD, Leung WC. The functions of breast cancer susceptibility gene 1 (BRCA1) product and its associated proteins. Biol Chem. 1999 Feb;380(2):117-28.

Isackson PJ, Fishback JL, Bidney DL, Reeck GR. Preferential affinity of high molecular weight high mobility group non-histone chromatin proteins for single-stranded DNA. J Biol Chem. 1979 Jul 10;254(13):5569-72.

Ishikawa T, Ali-Osman F. Glutathione-associated cis-diamminedichloroplatinum(II) metabolism and ATP-dependent efflux from

leukemia cells. Molecular characterization of glutathione-platinum complex and its biological significance. J Biol Chem. 1993 Sep 25;268(27):20116-25.

Ishikawa T, Bao JJ, Yamane Y, Akimaru K, Frindrich K, Wright CD, Kuo MT. Coordinated induction of MRP/GS-X pump and gamma-glutamylcysteine synthetase by heavy metals in human leukemia cells. J Biol Chem. 1996 Jun 21;271(25):14981-8.

Ishikawa T. The ATP-dependent glutathione S-conjugate export pump. Trends Biochem Sci. 1992 Nov;17(11):463-8.

Jachymczyk WJ, von Borstel RC, Mowat MR, Hastings PJ. Repair of interstrand cross-links in DNA of Saccharomyces cerevisiae requires two systems for DNA repair: the RAD3 system and the RAD51 system. Mol Gen Genet. 1981;182(2):196-205.

Jacquemier J, Penault-Llorca F, Viens P, Houvenaeghel G, Hassoun J, Torrente M, Adelaide J, Birnbaum D. Breast cancer response to adjuvant chemotherapy in correlation with erbB2 and p53 expression. Anticancer Res. 1994 Nov-Dec;14(6B):2773-8.

Jayaraman J, Prives C. Activation of p53 sequence-specific DNA binding by short single strands of DNA requires the p53 C-terminus. Cell. 1995 Jun 30;81(7):1021-9.

Johnson A, Qu Y, Van Houten B, Farrell N. B----Z DNA conformational changes induced by a family of dinuclear bis(platinum) complexes. Nucleic Acids Res. 1992 Apr 11;20(7):1697-703.

Johnson RD, Liu N, Jasin M. Mammalian XRCC2 promotes the repair of DNA double-strand breaks by homologous recombination. Nature. 1999 Sep 23;401(6751):397-9.

Johnson RD, Symington LS. Functional differences and interactions among the putative RecA homologs Rad51, Rad55, and Rad57. Mol Cell Biol. 1995 Sep;15(9):4843-50.

Johnson SW, Laub PB, Beesley JS, Ozols RF, Hamilton TC. Increased platinum-DNA damage tolerance is associated with cisplatin resistance and cross-resistance to various chemotherapeutic agents in unrelated human ovarian cancer cell lines. Cancer Res. 1997 Mar 1;57(5):850-6.

Johnson SW, Perez RP, Godwin AK, Yeung AT, Handel LM, Ozols RF, Hamilton TC. Role of platinum-DNA adduct formation and removal in cisplatin resistance in human ovarian cancer cell lines. Biochem Pharmacol. 1994 Feb 11;47(4):689-97.

Jones JC, Zhen WP, Reed E, Parker RJ, Sancar A, Bohr VA. Gene-specific formation and repair of cisplatin intrastrand adducts and interstrand cross-links in Chinese hamster ovary cells. J Biol Chem. 1991 Apr 15;266(11):7101-7.

Jones NA, Turner J, McIlwrath AJ, Brown R, Dive C. Cisplatin- and paclitaxel-induced apoptosis of ovarian carcinoma cells and the relationship between bax and bak up-regulation and the functional status of p53. Mol Pharmacol. 1998 May;53(5):819-26.

Jones SL, Hickson ID, Harris AL, Harnett PR. Repair of cisplatin-DNA adducts by protein extracts from human ovarian carcinoma. Int J Cancer. 1994 Nov 1;59(3):388-93.

Kadyk LC, Hartwell LH. Replication-dependent sister chromatid recombination in rad1 mutants of Saccharomyces cerevisiae. Genetics. 1993 Mar;133(3):469-87.

Kadyk LC, Hartwell LH. Sister chromatids are preferred over homologs as substrates for recombinational repair in Saccharomyces cerevisiae. Genetics. 1992 Oct;132(2):387-402.

Kanaar R, Hoeijmakers JH, van Gent DC. Molecular mechanisms of DNA double strand break repair. Trends Cell Biol. 1998 Dec;8(12):483-9.

Kao GD, McKenna WG, Muschel RJ. p34(Cdc2) kinase activity is excluded from the nucleus during the radiation-induced G(2) arrest in HeLa cells. J Biol Chem. 1999 Dec 3;274(49):34779-84.

Karran P, Bignami M. DNA damage tolerance, mismatch repair and genome instability. Bioessays. 1994 Nov;16(11):833-9.

Kasahara K, Fujiwara Y, Nishio K, Ohmori T, Sugimoto Y, Komiya K, Matsuda T, Saijo N. Metallothionein content correlates with the sensitivity of human small cell lung cancer cell lines to cisplatin. Cancer Res. 1991 Jun 15;51(12):3237-42.

Kashani-Sabet M, Rossi JJ, Lu Y, Ma JX, Chen J, Miyachi H, Scanlon KJ. Detection of drug resistance in human tumors by in vitro enzymatic amplification. Cancer Res. 1988 Oct 15;48(20):5775-8.

Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW. Participation of p53 protein in the cellular response to DNA damage. Cancer Res. 1991 Dec 1;51(23 Pt 1):6304-11.

Kastan MB, Zhan Q, el-Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B, Fornace AJ. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell. 1992 Nov 13;71(4):587-97.

Kat A, Thilly WG, Fang WH, Longley MJ, Li GM, Modrich P. An alkylation-tolerant, mutator human cell line is deficient in strand-specific mismatch repair. Proc Natl Acad Sci U S A. 1993 Jul 15;90(14):6424-8.

Kaufmann SH. Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. Cancer Res. 1989 Nov 1;49(21):5870-8 (a).

Kaufmann WK. Pathways of human cell post-replication repair. Carcinogenesis. 1989 Jan;10(1):1-11 (b).

Kelland LR, Abel G, McKeage MJ, Jones M, Goddard PM, Valenti M, Murrer BA, Harrap KR. Preclinical antitumor evaluation of bis-acetato-ammine-dichloro-cyclohexylamine platinum(IV): an orally active platinum drug. Cancer Res. 1993 Jun 1;53(11):2581-6 (a).

Kelland LR, McKeage MJ. New platinum agents. A comparison in ovarian cancer. Drugs Aging. 1994 Aug;5(2):85-95.

Kelland LR, Mistry P, Abel G, Freidlos F, Loh SY, Roberts JJ, Harrap KR. Establishment and characterization of an in vitro model of acquired resistance to cisplatin in a human testicular nonseminomatous germ cell line. Cancer Res. 1992 Apr 1;52(7):1710-6 (a).

Kelland LR, Murrer BA, Abel G, Giandomenico CM, Mistry P, Harrap KR. Ammine/amine platinum(IV) dicarboxylates: a novel class of platinum complex exhibiting selective cytotoxicity to intrinsically cisplatin-resistant human ovarian carcinoma cell lines. Cancer Res. 1992 Feb 15;52(4):822-8 (c).

Kelland LR, Murrer BA, Abel G, Giandomenico CM, Mistry P, Harrap KR. Ammine/amine platinum(IV) dicarboxylates: a novel class of platinum complex exhibiting selective cytotoxicity to intrinsically cisplatin-resistant human ovarian carcinoma cell lines. Cancer Res. 1992 Feb 15;52(4):822-8 (b).

Kelland LR. New platinum antitumor complexes. Crit Rev Oncol Hematol. 1993 Dec;15(3):191-219 (b).

Kelley SL, Basu A, Teicher BA, Hacker MP, Hamer DH, Lazo JS. Overexpression of metallothionein confers resistance to anticancer drugs. Science. 1988 Sep 30;241(4874):1813-5.

Kessis TD, Slebos RJ, Nelson WG, Kastan MB, Plunkett BS, Han SM, Lorincz AT, Hedrick L, Cho KR. Human papillomavirus 16 E6 expression disrupts

the p53-mediated cellular response to DNA damage. Proc Natl Acad Sci U S A. 1993 May 1;90(9):3988-92.

King RW, Jackson PK, Kirschner MW. Mitosis in transition. Cell. 1994 Nov 18;79(4):563-71.

Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science. 1997 Feb 21;275(5303):1132-6.

Koberle B, Grimaldi KA, Sunters A, Hartley JA, Kelland LR, Masters JR. DNA repair capacity and cisplatin sensitivity of human testis tumour cells. Int J Cancer. 1997 Mar 4;70(5):551-5.

Koberle B, Payne J, Grimaldi KA, Hartley JA, Masters JR. DNA repair in cisplatin-sensitive and resistant human cell lines measured in specific genes by quantitative polymerase chain reaction. Biochem Pharmacol. 1996 Dec 13;52(11):1729-34.

Kohn KW, Erickson LC, Ewig RA, Friedman CA. Fractionation of DNA from mammalian cells by alkaline elution. Biochemistry. 1976 Oct 19;15(21):4629-37.

Kohn KW, Jackman J, O'Connor PM. Cell cycle control and cancer chemotherapy. J Cell Biochem. 1994 Apr;54(4):440-52.

Koi M, Umar A, Chauhan DP, Cherian SP, Carethers JM, Kunkel TA, Boland CR. Human chromosome 3 corrects mismatch repair deficiency and microsatellite instability and reduces N-methyl-N'-nitro-N-nitrosoguanidine tolerance in colon tumor cells with homozygous hMLH1 mutation. Cancer Res. 1994 Aug 15;54(16):4308-12.

Koike K, Kawabe T, Tanaka T, Toh S, Uchiumi T, Wada M, Akiyama S, Ono M, Kuwano M. A canalicular multispecific organic anion transporter (cMOAT) antisense cDNA enhances drug sensitivity in human hepatic cancer cells. Cancer Res. 1997 Dec 15;57(24):5475-9.

Kolodner R. Biochemistry and genetics of eukaryotic mismatch repair. Genes Dev. 1996 Jun 15;10(12):1433-42.

Kondo S, Barnett GH, Hara H, Morimura T, Takeuchi J. MDM2 protein confers the resistance of a human glioblastoma cell line to cisplatin-induced apoptosis. Oncogene. 1995 May 18;10(10):2001-6.

Kosower NS, Kosower EM. The glutathione status of cells. Int Rev Cytol. 1978;54:109-60.

Kowalczykowski SC. In vitro reconstitution of homologous recombination reactions. Experientia. 1994 Mar 15;50(3):204-15.

Kuerbitz SJ, Plunkett BS, Walsh WV, Kastan MB. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc Natl Acad Sci U S A. 1992 Aug 15;89(16):7491-5.

Kunkel TA. DNA-mismatch repair. The intricacies of eukaryotic spell-checking. Curr Biol. 1995 Oct 1;5(10):1091-4.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970 Aug 15;227(259):680-5.

Larson JS, Tonkinson JL, Lai MT. A BRCA1 mutant alters G2-M cell cycle control in human mammary epithelial cells. Cancer Res. 1997 Aug 15;57(16):3351-5.

Lau CC, Pardee AB. Mechanism by which caffeine potentiates lethality of nitrogen mustard. Proc Natl Acad Sci U S A. 1982 May;79(9):2942-6.

Leach FS, Nicolaides NC, Papadopoulos N, Liu B, Jen J, Parsons R, Peltomaki P, Sistonen P, Aaltonen LA, Nystrom-Lahti M, et al. Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. Cell. 1993 Dec 17;75(6):1215-25.

Lee FY, Allalunis-Turner MJ, Siemann DW. Depletion of tumour versus normal tissue glutathione by buthionine sulfoximine. Br J Cancer. 1987 Jul;56(1):33-8.

Lee JS. Alternative dideoxy sequencing of double-stranded DNA by cyclic reactions using Taq polymerase. DNA Cell Biol. 1991 Jan-Feb;10(1):67-73.

Lee S, Elenbaas B, Levine A, Griffith J. p53 and its 14 Kda C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismathces. Cell. 1995 81:1013-20.

Levine AJ, Momand J, Finlay CA. The p53 tumour suppressor gene. Nature. 1991 Jun 6;351(6326):453-6.

Lewin B. Driving the cell cycle: M phase kinase, its partners, and substrates. Cell. 1990 Jun 1;61(5):743-52.

Li GM, Modrich P. Restoration of mismatch repair to nuclear extracts of H6 colorectal tumor cells by a heterodimer of human MutL homologs. Proc Natl Acad Sci U S A. 1995 Mar 14;92(6):1950-4.

Liang F, Han M, Romanienko PJ, Jasin M. Homology-directed repair is a major double-strand break repair pathway in mammalian cells. Proc Natl Acad Sci U S A. 1998 Apr 28;95(9):5172-7.

Liebermann DA, Hoffman B, Steinman RA. Molecular controls of growth arrest and apoptosis: p53-dependent and independent pathways. Oncogene. 1995 Jul 6;11(1):199-210.

Lilley DM. DNA--protein interactions. HMG has DNA wrapped up. Nature. 1992 May 28;357(6376):282-3.

Lin D, Fiscella M, O'Connor PM, Jackman J, Chen M, Luo LL, Sala A, Travali S, Appella E, Mercer WE. Constitutive expression of B-myb can bypass p53-induced Waf1/Cip1-mediated G1 arrest. Proc Natl Acad Sci U S A. 1994 Oct 11;91(21):10079-83.

Lin FL, Sperle K, Sternberg N. Model for homologous recombination during transfer of DNA into mouse L cells: role for DNA ends in the recombination process. Mol Cell Biol. 1984 Jun;4(6):1020-34.

Lin Y, Lukacsovich T, Waldman AS. Multiple pathways for repair of DNA double-strand breaks in mammalian chromosomes. Mol Cell Biol. 1999 Dec;19(12):8353-60.

Liu B, Parsons R, Papadopoulos N, Nicolaides NC, Lynch HT, Watson P, Jass JR, Dunlop M, Wyllie A, Peltomaki P, de la Chapelle A, Hamilton SR, Vogelstein B, Kinzler KW. Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients. Nat Med. 1996 Feb;2(2):169-74.

Liu L, Markowitz S, Gerson SL. Mismatch repair mutations override alkyltransferase in conferring resistance to temozolomide but not to 1,3-bis(2-chloroethyl)nitrosourea. Cancer Res. 1996 Dec 1;56(23):5375-9.

Livingstone LR, White A, Sprouse J, Livanos E, Jacks T, Tlsty TD. Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. Cell. 1992 Sep 18;70(6):923-35.

Lock RB, Keeling PK. Responses of HeLa and Chinese hamster ovary p34Cdc2/cyclin B1-kinase in relation to cell cycle perturbations induced by etoposide. Int J Oncol. 1993 3:33-42.

Loeb LA. Microsatellite instability: marker of a mutator phenotype in cancer. Cancer Res. 1994 Oct 1;54(19):5059-63.

Loehrer PJ, Einhorn LH. Drugs five years later. Cisplatin. Ann Intern Med. 1984 May;100(5):704-13.

Loh SY, Mistry P, Kelland LR, Abel G, Harrap KR. Reduced drug accumulation as a major mechanism of acquired resistance to cisplatin in a human ovarian carcinoma cell line: circumvention studies using novel platinum (II) and (IV) ammine/amine complexes. Br J Cancer. 1992 Dec;66(6):1109-15.

Lowe SW, Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell. 1993 Sep 24;74(6):957-67 (a).

Lowe SW, Schmitt EM, Smith SW, Osborne BA, Jacks T. p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature. 1993 Apr 29;362(6423):847-9 (b).

Lu Y, Han J, Scanlon KJ. Biochemical and molecular properties of cisplatin-resistant A2780 cells grown in folinic acid. J Biol Chem. 1988 Apr 5;263(10):4891-4.

Luthman M, Eriksson S, Holmgren A, Thelander L. Glutathione-dependent hydrogen donor system for calf thymus ribonucleoside-diphosphate reductase. Proc Natl Acad Sci U S A. 1979 May;76(5):2158-62.

Mack DH, Vartikar J, Pipas JM, Laimins LA. Specific repression of TATA-mediated but not initiator-mediated transcription by wild-type p53. Nature. 1993 May 20;363(6426):281-3.

Maity A, McKenna WG, Muschel RJ. Evidence for post-transcriptional regulation of cyclin B1 mRNA in the cell cycle and following irradiation in HeLa cells. EMBO J. 1995 Feb 1;14(3):603-9.

Mamenta EL, Poma EE, Kaufmann WK, Delmastro DA, Grady HL, Chaney SG. Enhanced replicative bypass of platinum-DNA adducts in cisplatin-resistant human ovarian carcinoma cell lines. Cancer Res. 1994 Jul 1;54(13):3500-5.

Mangioni C, Bolis G, Pecorelli S, Bragman K, Epis A, Favalli G, Gambino A, Landoni F, Presti M, Torri W, et al. Randomized trial in advanced ovarian cancer comparing cisplatin and carboplatin. J Natl Cancer Inst. 1989 Oct 4;81(19):1464-71.

Mann SC, Andrews PA, Howell SB. Short-term cis-diamminedichloroplatinum(II) accumulation in sensitive and resistant human ovarian carcinoma cells. Cancer Chemother Pharmacol. 1990;25(4):236-40.

Mannervik B, Danielson UH. Glutathione transferases--structure and catalytic activity. CRC Crit Rev Biochem. 1988;23(3):283-337.

Mansouri A, Henle KJ, Benson AM, Moss AJ, Nagle WA. Characterization of a cisplatin-resistant subline of murine RIF-1 cells and reversal of drug resistance by hyperthermia. Cancer Res. 1989 May 15;49(10):2674-8.

Manzotti C, Pratesi G, Menta E, Di Domenico R , Cavalletti E, Fiebig HH,Kelland LR, Farrell N, Polizzi D, Supino R, Pezzoni G, Zunino F. BBR 3464: a novel triplatinum complex, exhibiting a preclinical profile of antitumor efficacy different from cisplatin Clin Cancer Res. 2000 Jul;6(7):2626-34.

Markowitz S, Wang J, Myeroff L, Parsons R, Sun L, Lutterbaugh J, Fan RS, Zborowska E, Kinzler KW, Vogelstein B, et al. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. Science. 1995 Jun 2;268(5215):1336-8.

Marples B, Adomat H, Billings PC, Farrell NP, Koch CJ, Skov KA. Recognition of platinum-induced DNA damage by nuclear proteins: screening for mechanisms. Anticancer Drug Des. 1994 Oct;9(5):389-99.

Martinez J, Georgoff I, Martinez J, Levine AJ. Cellular localization and cell cycle regulation by a temperature-sensitive p53 protein. Genes Dev. 1991 Feb;5(2):151-9.

Masuda H, Tanaka T, Matsuda H, Kusaba I. Increased removal of DNA-bound platinum in a human ovarian cancer cell line resistant to cis-diamminedichloroplatinum(II). Cancer Res. 1990 Mar 15;50(6):1863-6.

Matsuyama S, Llopis J, Deveraux QL, Tsien RY, Reed JC. Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis. Nat Cell Biol. 2000 Jun;2(6):318-25.

May A, Nairn RS, Okumoto DS, Wassermann K, Stevnsner T, Jones JC, Bohr VA. Repair of individual DNA strands in the hamster dihydrofolate reductase gene after treatment with ultraviolet light, alkylating agents, and cisplatin. J Biol Chem. 1993 Jan 25;268(3):1650-7.

McANulty MM, Lippard SJ. The HMG-domain protein Ixr1 blocks excision repair of cisplatin-DNA adducts in yeast. Mutat Res. 1996 Jan 2;362(1):75-86 (a).

McANulty MM, Whitehead JP, Lippard SJ. Binding of Ixr1, a yeast HMG-domain protein, to cisplatin-DNA adducts in vitro and in vivo. Biochemistry. 1996 May 14;35(19):6089-99 (b).

McDonald ER, Wu GS, Waldman T, El-Deiry WS. Repair Defect in p21 WAF1/CIP1 -/- human cancer cells. Cancer Res. 1996 May 15;56(10):2250-5.

McKeage MJ, Mistry P, Ward J, Boxall FE, Loh S, O'Neill C, Ellis P, Kelland LR, Morgan SE, Murrer B, et al. A phase I and pharmacology study of an oral platinum complex, JM216: dose-dependent pharmacokinetics with single-dose administration. Cancer Chemother Pharmacol. 1995;36(6):451-8.

McKenna WG, Iliakis G, Weiss MC, Bernhard EJ, Muschel RJ. Increased G2 delay in radiation-resistant cells obtained by transformation of primary rat embryo cells with the oncogenes H-ras and v-myc. Radiat Res. 1991 Mar;125(3):283-7.

McLaughlin K, Coren G, Masters J, Brown R. Binding activities of cis-platin-damage-recognition proteins in human tumour cell lines. Int J Cancer. 1993 Feb 20;53(4):662-6.

Meijer C, Mulder NH, de Vries EG. The role of detoxifying systems in resistance of tumor cells to cisplatin and adriamycin. Cancer Treat Rev. 1990 Dec;17(4):389-407 (a).

Meijer C, Mulder NH, Hospers GA, Uges DR, de Vries EG. The role of glutathione in resistance to cisplatin in a human small cell lung cancer cell line. Br J Cancer. 1990 Jul;62(1):72-7 (b).

Meister A, Anderson ME. Glutathione. Annu Rev Biochem. 1983;52:711-60.

Melendez-Zajgla J, Cruz E, Maldonado V, Espinoza AM. Mitochondrial changes during the apoptotic process of HeLa cells exposed to cisplatin. Biochem Mol Biol Int. 1999 May;47(5):765-71.

Mellish KJ, Kelland LR, Harrap KR. In vitro platinum drug chemosensitivity of human cervical squamous cell carcinoma cell lines with intrinsic and acquired resistance to cisplatin. Br J Cancer. 1993 Aug;68(2):240-50.

Mello JA, Acharya S, Fishel R, Essigmann JM. The mismatch-repair protein hMSH2 binds selectively to DNA adducts of the anticancer drug cisplatin. Chem Biol. 1996 Jul;3(7):579-89.

Mellon I, Champe GN. Products of DNA mismatch repair genes mutS and mutL are required for transcription-coupled nucleotide-excision repair of the lactose operon in Escherichia coli. Proc Natl Acad Sci U S A. 1996 Feb 6;93(3):1292-7.

Mellon I, Rajpal DK, Koi M, Boland CR, Champe GN. Transcription-coupled repair deficiency and mutations in human mismatch repair genes. Science. 1996 Apr 26;272(5261):557-60.

Merlo A, Mabry M, Gabrielson E, Vollmer R, Baylin SB, Sidransky D. Frequent microsatellite instability in primary small cell lung cancer. Cancer Res. 1994 Apr 15;54(8):2098-101.

Metting NF, Little JB. Transient failure to dephosphorylate the cdc2-cyclin B1 complex accompanies radiation-induced G2-phase arrest in HeLa cells. Radiat Res. 1995 Sep;143(3):286-92.

Micetich K, Zwelling LA, Kohn KW. Quenching of DNA:platinum(II) monoadducts as a possible mechanism of resistance to cis-diamminedichloroplatinum(II) in L1210 cells. Cancer Res. 1983 Aug;43(8):3609-13.

Michalovitz D, Halevy O, Oren M. Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. Cell. 1990 Aug 24;62(4):671-80.

Milner J, Ponder B, Hughes-Davies L, Seltmann M, Kouzarides T. Transcriptional activation functions in BRCA2. Nature. 1997 Apr 24;386(6627):772-3.

Mistry P, Kelland LR, Abel G, Sidhar S, Harrap KR. The relationships between glutathione, glutathione-S-transferase and cytotoxicity of platinum drugs and melphalan in eight human ovarian carcinoma cell lines. Br J Cancer. 1991 Aug;64(2):215-20.

Mistry P, Kelland LR, Loh SY, Abel G, Murrer BA, Harrap KR. Comparison of cellular accumulation and cytotoxicity of cisplatin with that of tetraplatin and amminedibutyratodichloro(cyclohexylamine)platinum(IV) (JM221) in

human ovarian carcinoma cell lines. Cancer Res. 1992 Nov 15;52(22):6188-93.

Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B, Reed JC. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene. 1994 Jun;9(6):1799-805.

Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell. 1995 Jan 27;80(2):293-9.

Modrich P. Mismatch repair, genetic stability, and cancer. Science. 1994 Dec 23;266(5193):1959-60.

Moggs JG, Szymkowski DE, Yamada M, Karran P, Wood RD. Differential human nucleotide excision repair of paired and mispaired cisplatin-DNA adducts. Nucleic Acids Res. 1997 Feb 1;25(3):480-91.

Monteiro AN, August A, Hanafusa H. Evidence for a transcriptional activation function of BRCA1 C-terminal region. Proc Natl Acad Sci U S A. 1996 Nov 26;93(24):13595-9.

Moynahan ME, Chiu JW, Koller BH, Jasin M. Brca1 controls homology-directed DNA repair. Mol Cell. 1999 Oct;4(4):511-8.

Mu D, Hsu DS, Sancar A. Reaction mechanism of human DNA repair excision nuclease. J Biol Chem. 1996 Apr 5;271(14):8285-94.

Murray AW. Creative blocks: cell-cycle checkpoints and feedback controls. Nature. 1992 Oct 15;359(6396):599-604.

Murray V, Motyka H, England PR, Wickham G, Lee HH, Denny WA, McFadyen WD. An investigation of the sequence-specific interaction of cis-diamminedichloroplatinum(II) and four analogues, including two acridine-

tethered complexes, with DNA inside human cells. Biochemistry. 1992 Dec 1;31(47):11812-7.

Muschel RJ, Zhang HB, Iliakis G, McKenna WG. Cyclin B expression in HeLa cells during the G2 block induced by ionizing radiation. Cancer Res. 1991 Oct 1;51(19):5113-7.

Muschel RJ, Zhang HB, McKenna WG. Differential effect of ionizing radiation on the expression of cyclin A and cyclin B in HeLa cells. Cancer Res. 1993 Mar 1;53(5):1128-35.

Nakagawa K, Saijo N, Tsuchida S, Sakai M, Tsunokawa Y, Yokota J, Muramatsu M, Sato K, Terada M, Tew KD. Glutathione-S-transferase pi as a determinant of drug resistance in transfectant cell lines. J Biol Chem. 1990 Mar 15;265(8):4296-301.

Naredi P, Heath DD, Enns RE, Howell SB. Cross-resistance between cisplatin, antimony potassium tartrate, and arsenite in human tumor cells. J Clin Invest. 1995 Mar;95(3):1193-8.

Nehme A, Baskaran R, Aebi S, Fink D, Nebel S, Cenni B, Wang JY, Howell SB, Christen RD. Differential induction of c-Jun NH2-terminal kinase and c-Abl kinase in DNA mismatch repair-proficient and -deficient cells exposed to cisplatin. Cancer Res. 1997 Aug 1;57(15):3253-7.

Nelson WG, Kastan MB. DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. Mol Cell Biol. 1994 Mar;14(3):1815-23.

Nicolaides NC, Papadopoulos N, Liu B, Wei YF, Carter KC, Ruben SM, Rosen CA, Haseltine WA, Fleischmann RD, Fraser CM, et al. Mutations of

two PMS homologues in hereditary nonpolyposis colon cancer. Nature. 1994 Sep 1;371(6492):75-80.

Nishio K, Fujiwara Y, Miyahara Y, Takeda Y, Ohira T, Kubota N, Ohta S, Funayama Y, Ogasawara H, Ohata M, et al. Cis-diamminedichloroplatinum(II) inhibits p34cdc2 protein kinase in human lung-cancer cells. Int J Cancer. 1993 Oct 21;55(4):616-22.

Oberhammer FA, Hochegger K, Froschl G, Tiefenbacher R, Pavelka M. Chromatin condensation during apoptosis is accompanied by degradation of lamin A+B, without enhanced activation of cdc2 kinase. J Cell Biol. 1994 Aug;126(4):827-37.

O'Connor PM, Fan S. DNA damage checkpoints: implications for cancer therapy. Prog Cell Cycle Res. 1996;2:165-73.

O'Connor PM, Ferris DK, Hoffmann I, Jackman J, Draetta G, Kohn KW. Role of the cdc25C phosphatase in G2 arrest induced by nitrogen mustard. Proc Natl Acad Sci U S A. 1994 Sep 27;91(20):9480-4.

O'Connor PM, Ferris DK, Pagano M, Draetta G, Pines J, Hunter T, Longo DL, Kohn KW. G2 delay induced by nitrogen mustard in human cells affects cyclin A/cdk2 and cyclin B1/cdc2-kinase complexes differently. J Biol Chem. 1993 Apr 15;268(11):8298-308 (a).

O'Connor PM, Jackman J, Jondle D, Bhatia K, Magrath I, Kohn KW. Role of the p53 tumor suppressor gene in cell cycle arrest and radiosensitivity of Burkitt's lymphoma cell lines. Cancer Res. 1993 Oct 15;53(20):4776-80 (b).

O'Connor PM, Kohn KW. A fundamental role for cell cycle regulation in the chemosensitivity of cancer cells? Semin Cancer Biol. 1992 Dec;3(6):409-16.

O'Donovan A, Wood RD. Identical defects in DNA repair in xeroderma pigmentosum group G and rodent ERCC group 5. Nature. 1993 May 13;363(6425):185-8.

Ogawa T, Shinohara A, Nabetani A, Ikeya T, Yu X, Egelman EH, Ogawa H. RecA-like recombination proteins in eukaryotes: functions and structures of RAD51 genes. Cold Spring Harb Symp Quant Biol. 1993;58:567-76.

Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell. 1993 Aug 27;74(4):609-19.

O'Neill CF, Koberle B, Masters JR, Kelland LR. Gene-specific repair of Pt/DNA lesions and induction of apoptosis by the oral platinum drug JM216 in three human ovarian carcinoma cell lines sensitive and resistant to cisplatin. Br J Cancer. 1999 Dec;81(8):1294-303.

Orlandi L, Colella G, Bearzatto A, Abolafio G, Manzotti C, Daidone MG, Zaffaroni N. Effects of a novel trinuclear platinum complex in cisplatin-sensitive and cisplatin-resistant human ovarian cancer cell lines: interference with cell cycle progression and induction of apoptosis. Eur J Cancer. 2001 Mar;37(5):649-59.

Ormerod MG, O'Neill C, Robertson D, Kelland LR, Harrap KR. cis-Diamminedichloroplatinum(II)-induced cell death through apoptosis in sensitive and resistant human ovarian carcinoma cell lines. Cancer Chemother Pharmacol. 1996;37(5):463-71.

Ormerod MG, Orr RM, Peacock JH. The role of apoptosis in cell killing by cisplatin: a flow cytometric study. Br J Cancer. 1994 Jan;69(1):93-100.

Otto AM, Paddenberg R, Schubert S, Mannherz HG. Cell-cycle arrest, micronucleus formation, and cell death in growth inhibition of MCF-7 breast cancer cells by tamoxifen and cisplatin. J Cancer Res Clin Oncol. 1996;122(10):603-12.

Ouchi T, Monteiro AN, August A, Aaronson SA, Hanafusa H. BRCA1 regulates p53-dependent gene expression. Proc Natl Acad Sci U S A. 1998 Mar 3;95(5):2302-6.

Ozols RF, Young RC. Chemotherapy of ovarian cancer. Semin Oncol. 1991 Jun;18(3):222-32.

Palombo F, Gallinari P, Iaccarino I, Lettieri T, Hughes M, D'Arrigo A, Truong O, Hsuan JJ, Jiricny J. GTBP, a 160-kilodalton protein essential for mismatch-binding activity in human cells. Science. 1995 Jun 30;268(5219):1912-4.

Papadopoulos N, Nicolaides NC, Wei YF, Ruben SM, Carter KC, Rosen CA, Haseltine WA, Fleischmann RD, Fraser CM, Adams MD, et al. Mutation of a mutL homolog in hereditary colon cancer. Science. 1994 Mar 18;263(5153):1625-9.

Parker RJ, Eastman A, Bostick-Bruton F, Reed E. Acquired cisplatin resistance in human ovarian cancer cells is associated with enhanced repair of cisplatin-DNA lesions and reduced drug accumulation. J Clin Invest. 1991 Mar;87(3):772-7.

Parsons R, Li GM, Longley MJ, Fang WH, Papadopoulos N, Jen J, de la Chapelle A, Kinzler KW, Vogelstein B, Modrich P. Hypermutability and

mismatch repair deficiency in RER+ tumor cells. Cell. 1993 Dec 17;75(6):1227-36.

Patel KJ, Yu VP, Lee H, Corcoran A, Thistlethwaite FC, Evans MJ, Colledge WH, Friedman LS, Ponder BA, Venkitaraman AR. Involvement of Brca2 in DNA repair. Mol Cell. 1998 Feb;1(3):347-57.

Pegg AE, Boosalis M, Samson L, Moschel RC, Byers TL, Swenn K, Dolan ME. Mechanism of inactivation of human O6-alkylguanine-DNA alkyltransferase by O6-benzylguanine. Biochemistry. 1993 Nov 16;32(45):11998-2006.

Pendyala L, Creaven PJ. In vitro cytotoxicity, protein binding, red blood cell partitioning, and biotransformation of oxaliplatin. Cancer Res. 1993 Dec 15;53(24):5970-6.

Perego P, Caserini C, Gatti L, Carenini N, Romanelli S, Supino R, Colangelo D, Viano I, Leone R, Spinelli S, Pezzoni G, Manzotti C, Farrell N, Zunino F. A novel trinuclear platinum complex overcomes cisplatin resistance in an osteosarcoma cell system. Mol Pharmacol. 1999 Mar;55(3):528-34.

Perego P, Righetti SC, Supino R, Delia D, Caserini C, Carenini N, Bedognè B, Broome E, Krajewski S, Reed JC, Zunino F. Role of apoptosis and apoptosis-related proteins in the cisplatin-resistant phenotype of human tumor cell lines. 1997; 2(6): 540-548.

Pestell KE, Hobbs SM, Titley JC, Kelland LR, Walton MI. Effect of p53 status on sensitivity to platinum complexes in a human ovarian cancer cell line. Mol Pharmacol. 2000 Mar;57(3):503-11.

Philips DR and Cullinane C In: Methods in Molecular Biology: Drug-DNA Interaction Protocols. Fox KR (ed) 1997.Vol. 90: pp. 127-146: Human Press Inc., Totowa, NU USA.

Pierce AJ, Johnson RD, Thompson LH, Jasin M. XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. Genes Dev. 1999 Oct 15;13(20):2633-8.

Pietenpol JA, Tokino T, Thiagalingam S, el-Deiry WS, Kinzler KW, Vogelstein B. Sequence-specific transcriptional activation is essential for growth suppression by p53. Proc Natl Acad Sci U S A. 1994 Mar 15;91(6):1998-2002.

Pil PM, Lippard SJ. Specific binding of chromosomal protein HMG1 to DNA damaged by the anticancer drug cisplatin. Science. 1992 Apr 10;256(5054):234-7.

Pines J, Hunter T. Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cdc2. Cell. 1989 Sep 8;58(5):833-46.

Plumb JA, Strathdee G, Sludden J, Kaye SB, Brown R. Reversal of drug resistance in human tumor xenografts by 2'-deoxy-5-azacytidine-induced demethylation of the hMLH1 gene promoter. Cancer Res. 2000 Nov 1;60(21):6039-44.

Ponti M, Forrow SM, Souhami RL, D'Incalci M, Hartley JA. Measurement of the sequence specificity of covalent DNA modification by antineoplastic agents using Taq DNA polymerase. Nucleic Acids Res. 1991 Jun 11;19(11):2929-33

Powell SN, DeFrank JS, Connell P, Eogan M, Preffer F, Dombkowski D, Tang W, Friend S. Differential sensitivity of p53(-) and p53(+) cells to caffeine-induced radiosensitization and override of G2 delay. Cancer Res. 1995 Apr 15;55(8):1643-8.

Pratesi G, Giuliani FC, Polizzi D, Righetti S, Manzotti C, Pezzoni G, Farrell N, Zunino F. Complete lack of cross-resistance of cisplatin-resistant tumors to a novel charged trinuclear platinum complex: p53-independent antitumor efficacy. Proc Am Assoc Cancer Res 1997; 38: 310.

Pratesi G, Perego P, Polizzi D, Righetti SC, Supino R, Caserini C, Manzotti C, Giuliani FC, Pezzoni G, Tognella S, Spinelli S, Farrell N, Zunino F. A novel charged trinuclear platinum complex effective against cisplatin-resistant tumours: hypersensitivity of p53-mutant human tumour xenografts. Br J Cancer. 1999 Aug;80(12):1912-9.

Prestayko AW, D'Aoust JC, Issell BF, Crooke ST. Cisplatin (cis-diamminedichloroplatinum II). Cancer Treat Rev. 1979 Mar;6(1):17-39.

Price A. The repair of ionising radiation-induced damage to DNA. Semin Cancer Biol. 1993 Apr;4(2):61-71.

Qin XQ, Livingston DM, Kaelin WG, Adams PD. Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. Proc Natl Acad Sci U S A. 1994 Nov 8;91(23):10918-22.

Rampino N, Yamamoto H, Ionov Y, Li Y, Sawai H, Reed JC, Perucho M. Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. Science. 1997 Feb 14;275(5302):967-9.

Reed E. The encyclopaedia of cancer. JR Bertino (ed.). 1996. pp. 1192-1200: Academic Press, San Diego, CA USA.

Reitmair AH, Schmits R, Ewel A, Bapat B, Redston M, Mitri A, Waterhouse P, Mittrucker HW, Wakeham A, Liu B, et al. MSH2 deficient mice are viable and susceptible to lymphoid tumours. Nat Genet. 1995 Sep;11(1):64-70.

Richon VM, Schulte N, Eastman A. Multiple mechanisms of resistance to cis-diamminedichloroplatinum(II) in murine leukemia L1210 cells. Cancer Res. 1987 Apr 15;47(8):2056-61.

Righetti SC, Della Torre G, Pilotti S, Menard S, Ottone F, Colnaghi MI, Pierotti MA, Lavarino C, Cornarotti M, Oriana S, Bohm S, Bresciani GL, Spatti G, Zunino F. A comparative study of p53 gene mutations, protein accumulation, and response to cisplatin-based chemotherapy in advanced ovarian carcinoma. Cancer Res. 1996 Feb 15;56(4):689-93.

Ringborg U, Hansson J, Jungnelius U, Berhane K, Castro V, Mannerwik B. Ethacrynic acid (EA) inhibition of glutathione transferases (GSTs) as a mechanism for enhanced melphalan toxicity in human melanoma. Proc Am Assoc Cancer Res 1990; 31: 2190.

Risinger JI, Berchuck A, Kohler MF, Watson P, Lynch HT, Boyd J. Genetic instability of microsatellites in endometrial carcinoma. Cancer Res. 1993 Nov 1;53(21):5100-3.

Risinger JI, Umar A, Barrett JC, Kunkel TA. A hPMS2 mutant cell line is defective in strand-specific mismatch repair. J Biol Chem. 1995 Aug 4;270(31):18183-6.

Risinger JI, Umar A, Boyd J, Berchuck A, Kunkel TA, Barrett JC. Mutation of MSH3 in endometrial cancer and evidence for its functional role in heteroduplex repair. Nat Genet. 1996 Sep;14(1):102-5.

Rixe O, Ortuzar W, Alvarez M, Parker R, Reed E, Paull K, Fojo T. Oxaliplatin, tetraplatin, cisplatin, and carboplatin: spectrum of activity in drug-resistant cell lines and in the cell lines of the National Cancer Institute's Anticancer Drug Screen panel. Biochem Pharmacol. 1996 Dec 24;52(12):1855-65.

Robson CN, Lewis AD, Wolf CR, Hayes JD, Hall A, Proctor SJ, Harris AL, Hickson ID. Reduced levels of drug-induced DNA cross-linking in nitrogen mustard-resistant Chinese hamster ovary cells expressing elevated glutathione S-transferase activity. Cancer Res. 1987 Nov 15;47(22):6022-7.

Royer-Pokora B, Gordon LK, Haseltine WA. Use of exonuclease III to determine the site of stable lesions in defined sequences of DNA: the cyclobutane pyrimidine dimer and cis and trans dichlorodiammine platinum II examples. Nucleic Acids Res. 1981 Sep 25;9(18):4595-609.

Russell KJ, Wiens LW, Demers GW, Galloway DA, Plon SE, Groudine M. Abrogation of the G2 checkpoint results in differential radiosensitization of G1 checkpoint-deficient and G1 checkpoint-competent cells. Cancer Res. 1995 Apr 15;55(8):1639-42.

Saburi Y, Nakagawa M, Ono M, Sakai M, Muramatsu M, Kohno K, Kuwano M. Increased expression of glutathione S-transferase gene in cis-diamminedichloroplatinum(II)-resistant variants of a Chinese hamster ovary cell line. Cancer Res. 1989 Dec 15;49(24 Pt 1):7020-5.

Salles B, Frit P, Provot C, Jaeg JP, Calsou P. In vitro eukaryotic DNA excision repair assays: an overview. Biochimie. 1995;77(10):796-802.

Salvioli S, Ardizzoni A, Franceschi C, Cossarizza A. JC-1, but not DiOC6(3) or rhodamine 123, is a reliable fluorescent probe to assess delta psi changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. FEBS Lett. 1997 Jul 7;411(1):77-82.

Sancar A. DNA excision repair. Annu Rev Biochem. 1996;65:43-81.

Sancar A. DNA repair in humans. Annu Rev Genet. 1995;29:69-105.

Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A. 1977 Dec;74(12):5463-7.

Savitsky K, Bar-Shira A, Gilad S, Rotman G, Ziv Y, Vanagaite L, Tagle DA, Smith S, Uziel T, Sfez S, et al A single ataxia telangiectasia gene with a product similar to PI-3 kinase. Science. 1995 Jun 23;268(5218):1749-53.

Scanlon KJ, Kashani-Sabet M, Miyachi H, Sowers LC, Rossi J. Molecular basis of cisplatin resistance in human carcinomas: model systems and patients. Anticancer Res. 1989 Sep-Oct;9(5):1301-12 (a).

Scanlon KJ, Kashani-Sabet M, Miyachi H. Differential gene expression in human cancer cells resistant to cisplatin. Cancer Invest. 1989;7(6):581-7 (b).

Scanlon KJ, Kashani-Sabet M. Elevated expression of thymidylate synthase cycle genes in cisplatin-resistant human ovarian carcinoma A2780 cells. Proc Natl Acad Sci U S A. 1988 Feb;85(3):650-3.

Scanlon KJ, Kashani-Sabet M. Utility of the polymerase chain reaction in detection of gene expression in drug-resistant human tumors. J Clin Lab Anal. 1989;3(5):323-9.

Scanlon KJ, Newman EM, Lu Y, Priest DG. Biochemical basis for cisplatin and 5-fluorouracil synergism in human ovarian carcinoma cells. Proc Natl Acad Sci U S A. 1986 Dec;83(23):8923-5.

Scanlon KJ, Safirstein RL, Thies H, Gross RB, Waxman S, Guttenplan JB. Inhibition of amino acid transport by cis-diamminedichloroplatinum(II) derivatives in L1210 murine leukemia cells. Cancer Res. 1983 Sep;43(9):4211-5.

Schilder RJ, Hall L, Monks A, Handel LM, Fornace AJ, Ozols RF, Fojo AT, Hamilton TC. Metallothionein gene expression and resistance to cisplatin in human ovarian cancer. Int J Cancer. 1990 Mar 15;45(3):416-22.

Scully R, Chen J, Ochs RL, Keegan K, Hoekstra M, Feunteun J, Livingston DM. Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. Cell. 1997 Aug 8;90(3):425-35.

Sekiya S, Oosaki T, Andoh S, Suzuki N, Akaboshi M, Takamizawa H. Mechanisms of resistance to cis-diamminedichloroplatinum (II) in a rat ovarian carcinoma cell line. Eur J Cancer Clin Oncol. 1989 Mar;25(3):429-37.

Selvakumaran M, Lin HK, Miyashita T, Wang HG, Krajewski S, Reed JC, Hoffman B, Liebermann D. Immediate early up-regulation of bax expression by p53 but not TGF beta 1: a paradigm for distinct apoptotic pathways. Oncogene. 1994 Jun;9(6):1791-8.

Sharan SK, Morimatsu M, Albrecht U, Lim DS, Regel E, Dinh C, Sands A, Eichele G, Hasty P, Bradley A. Embryonic lethality and radiation

hypersensitivity mediated by Rad51 in mice lacking Brca2. Nature. 1997 Apr 24;386(6627):804-10.

Sheibani N, Jennerwein MM, Eastman A. DNA repair in cells sensitive and resistant to cis-diamminedichloroplatinum(II): host cell reactivation of damaged plasmid DNA. Biochemistry. 1989 Apr 4;28(7):3120-4.

Shen D, Pastan I, Gottesman MM. Cross-resistance to methotrexate and metals in human cisplatin-resistant cell lines results from a pleiotropic defect in accumulation of these compounds associated with reduced plasma membrane binding proteins. Cancer Res. 1998 Jan 15;58(2):268-75.

Shimizu T, Cao CX, Shao RG, Pommier Y. Lamin B phosphorylation by protein kinase calpha and proteolysis during apoptosis in human leukemia HL60 cells. J Biol Chem. 1998 Apr 10;273(15):8669-74

Shimizu T, Pommier Y. Camptothecin-induced apoptosis in p53-null human leukemia HL60 cells and their isolated nuclei: effects of the protease inhibitors Z-VAD-fmk and dichloroisocoumarin suggest an involvement of both caspases and serine proteases. Leukemia. 1997 Aug;11(8):1238-44.

Shinohara A, Ogawa H, Ogawa T. Rad51 protein involved in repair and recombination in S. cerevisiae is a RecA-like protein. Cell. 1992 May 1;69(3):457-70.

Shinohara A, Ogawa T. Homologous recombination and the roles of double-strand breaks. Trends Biochem Sci. 1995 Oct;20(10):387-91.

Shivji KK, Kenny MK, Wood RD. Proliferating cell nuclear antigen is required for DNA excision repair. Cell. 1992 Apr 17;69(2):367-74.

Shridhar V, Siegfried J, Hunt J, del Mar Alonso M, Smith DI. Genetic instability of microsatellite sequences in many non-small cell lung carcinomas. Cancer Res. 1994 Apr 15;54(8):2084-7.

Siede W, Friedl AA, Dianova I, Eckardt-Schupp F, Friedberg EC. The Saccharomyces cerevisiae Ku autoantigen homologue affects radiosensitivity only in the absence of homologous recombination. Genetics. 1996 Jan;142(1):91-102.

Slichenmyer WJ, Nelson WG, Slebos RJ, Kastan MB. Loss of a p53-associated G1 checkpoint does not decrease cell survival following DNA damage. Cancer Res. 1993 Sep 15;53(18):4164-8.

Smith ML, Kontny HU, Zhan Q, Sreenath A, O'Connor PM, Fornace AJ. Antisense GADD45 expression results in decreased DNA repair and sensitizes cells to u.v.-irradiation or cisplatin. Oncogene. 1996 Nov 21;13(10):2255-63.

Smith-Sorensen B, Kaern J, Holm R, Dorum A, Trope C, Borresen-Dale AL. Therapy effect of either paclitaxel or cyclophosphamide combination treatment in patients with epithelial ovarian cancer and relation to TP53 gene status. Br J Cancer. 1998 Aug;78(3):375-81.

Snouwaert JN, Gowen LC, Latour AM, Mohn AR, Xiao A, DiBiase L, Koller BH. BRCA1 deficient embryonic stem cells display a decreased homologous recombination frequency and an increased frequency of non-homologous recombination that is corrected by expression of a brca1 transgene. Oncogene. 1999 Dec 20;18(55):7900-7.

Sobol RW, Horton JK, Kuhn R, Gu H, Singhal RK, Prasad R, Rajewsky K, Wilson SH. Requirement of mammalian DNA polymerase-beta in base-excision repair. Nature. 1996 Jan 11;379(6561):183-6.

Sonoda E, Sasaki MS, Buerstedde JM, Bezzubova O, Shinohara A, Ogawa H, Takata M, Yamaguchi-Iwai Y, Takeda S. Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death. EMBO J. 1998 Jan 15;17(2):598-608.

Sorenson CM, Barry MA, Eastman A. Analysis of events associated with cell cycle arrest at G2 phase and cell death induced by cisplatin. J Natl Cancer Inst. 1990 May 2;82(9):749-55.

Sorenson CM, Eastman A. Mechanism of cis-diamminedichloroplatinum(II)-induced cytotoxicity: role of G2 arrest and DNA double-strand breaks. Cancer Res. 1988 Aug 15;48(16):4484-8.

Souza RF, Appel R, Yin J, Wang S, Smolinski KN, Abraham JM, Zou TT, Shi YQ, Lei J, Cottrell J, Cymes K, Biden K, Simms L, Leggett B, Lynch PM, Frazier M, Powell SM, Harpaz N, Sugimura H, Young J, Meltzer SJ. Microsatellite instability in the insulin-like growth factor II receptor gene in gastrointestinal tumours. Nat Genet. 1996 Nov;14(3):255-7.

Souza RF, Yin J, Smolinski KN, Zou TT, Wang S, Shi YQ, Rhyu MG, Cottrell J, Abraham JM, Biden K, Simms L, Leggett B, Bova GS, Frank T, Powell SM, Sugimura H, Young J, Harpaz N, Shimizu K, Matsubara N, Meltzer SJ. Frequent mutation of the E2F-4 cell cycle gene in primary human gastrointestinal tumors. Cancer Res. 1997 Jun 15;57(12):2350-3.

Stewart N, Hicks GG, Paraskevas F, Mowat M. Evidence for a second cell cycle block at G2/M by p53. Oncogene. 1995 Jan 5;10(1):109-15.

Strathdee G, MacKean MJ, Illand M, Brown R. A role for methylation of the hMLH1 promoter in loss of hMLH1 expression and drug resistance in ovarian cancer. Oncogene. 1999 Apr 8;18(14):2335-41.

Su SS, Modrich P. Escherichia coli mutS-encoded protein binds to mismatched DNA base pairs. Proc Natl Acad Sci U S A. 1986 Jul;83(14):5057-61.

Sugimoto C, Fujieda S, Seki M, Sunaga H, Fan GK, Tsuzuki H, Borner C, Saito H, Matsukawa S. Apoptosis-promoting gene (bax) transfer potentiates sensitivity of squamous cell carcinoma to cisplatin in vitro and in vivo. Int J Cancer. 1999 Sep 9;82(6):860-7.

Sullivan DT. DNA excision repair and transcription: implications for genome evolution. Curr Opin Genet Dev. 1995 Dec;5(6):786-91.

Sung P. Yeast Rad55 and Rad57 proteins form a heterodimer that functions with replication protein A to promote DNA strand exchange by Rad51 recombinase. Genes Dev. 1997 May 1;11(9):1111-21.

Sunters A, Grimaldi KA, Souhami RL, Hartley JA. The use of alpha-DNA as an internal standard in the detection and quantitation of DNA damage in specific genes using Southern blotting. Nucleic Acids Res. 1996 Jun 15;24(12):2456-7.

Sweigert SE, Carroll D. Repair and recombination of X-irradiated plasmids in Xenopus laevis oocytes. Mol Cell Biol. 1990 Nov;10(11):5849-56.

Szostak JW, Orr-Weaver TL, Rothstein RJ, Stahl FW. The double-strand-break repair model for recombination. Cell. 1983 May;33(1):25-35.

Szymkowski DE, Yarema K, Essigmann JM, Lippard SJ, Wood RD. An intrastrand d(GpG) platinum crosslink in duplex M13 DNA is refractory to repair by human cell extracts. Proc Natl Acad Sci U S A. 1992 Nov 15;89(22):10772-6.

Takata M, Sasaki MS, Sonoda E, Fukushima T, Morrison C, Albala JS, Swagemakers SM, Kanaar R, Thompson LH, Takeda S. The Rad51 paralog Rad51B promotes homologous recombinational repair. Mol Cell Biol. 2000 Sep;20(17):6476-82.

Tashiro T, Kawada Y, Sakurai Y, Kidani Y. Antitumor activity of a new platinum complex, oxalato(trans-l-1,2-diaminocyclohexane)platinum (II): new experimental data. Biomed Pharmacother. 1989;43(4):251-60.

Taverna P, Hansson J, Scanlon KJ, Hill BT. Gene expression in X-irradiated human tumour cell lines expressing cisplatin resistance and altered DNA repair capacity. Carcinogenesis. 1994 Sep;15(9):2053-6.

Tavtigian SV, Simard J, Rommens J, Couch F, Shattuck-Eidens D, Neuhausen S, Merajver S, Thorlacius S, Offit K, Stoppa-Lyonnet D, Belanger C, Bell R, Berry S, Bogden R, Chen Q, Davis T, Dumont M, Frye C, Hattier T, Jammulapati S, Janecki T, Jiang P, Kehrer R, Leblanc JF, Goldgar DE, et al. The complete BRCA2 gene and mutations in chromosome 13q-linked kindreds. Nat Genet. 1996 Mar;12(3):333-7.

Teicher BA, Holden SA, Kelley MJ, Shea TC, Cucchi CA, Rosowsky A, Henner WD, Frei E. Characterization of a human squamous carcinoma cell line resistant to cis-diamminedichloroplatinum(II). Cancer Res. 1987 Jan 15;47(2):388-93.

Tew KD, Bomber AM, Hoffman SJ. Ethacrynic acid and piriprost as enhancers of cytotoxicity in drug resistant and sensitive cell lines. Cancer Res. 1988 Jul 1;48(13):3622-5.

Thacker J. A surfeit of RAD51-like genes? Trends Genet. 1999 May;15(5):166-8.

Thibodeau SN, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon. Science. 1993 May 7;260(5109):816-9.

Thiebaut F, Enns R, Howell SB. Cisplatin sensitivity correlates with its ability to cause cell cycle arrest via a wee1 kinase-dependent pathway in Schizosaccharomyces pombe. J Cell Physiol. 1994 Jun;159(3):506-14.

Thompson LH, Schild D. The contribution of homologous recombination in preserving genome integrity in mammalian cells. Biochimie. 1999 Jan-Feb;81(1-2):87-105.

Timmer-Bosscha H, Mulder NH, de Vries EG. Modulation of cis-diamminedichloroplatinum(II) resistance. Br J Cancer. 1992 Aug;66(2):227-38.

Tomlinson IP, Novelli MR, Bodmer WF. The mutation rate and cancer. Proc Natl Acad Sci U S A. 1996 Dec 10;93(25):14800-3.

Toney JH, Donahue BA, Kellett PJ, Bruhn SL, Essigmann JM, Lippard SJ. Isolation of cDNAs encoding a human protein that binds selectively to DNA modified by the anticancer drug cis-diamminedichloroplatinum(II) Proc Natl Acad Sci U S A. 1989 Nov;86(21):8328-32.

Twentyman PR, Wright KA, Mistry P, Kelland LR, Murrer BA. Sensitivity to novel platinum compounds of panels of human lung cancer cell lines with

acquired and inherent resistance to cisplatin. Cancer Res. 1992 Oct 15;52(20):5674-80.

Ueda K, Suzuki H, Akiyama S, Sugiyama Y. Differences in substrate specificity among glutathione conjugates (GS-X) pump family members: comparison between multidrug resistance-associated protein and a novel transporter expressed on a cisplatin-resistant cell line (KCP-4). Jpn J Cancer Res. 1999 Apr;90(4):439-47.

Umar A, Koi M, Risinger JI, Glaab WE, Tindall KR, Kolodner RD, Boland CR, Barrett JC, Kunkel TA. Correction of hypermutability, N-methyl-N'-nitro-N-nitrosoguanidine resistance, and defective DNA mismatch repair by introducing chromosome 2 into human tumor cells with mutations in MSH2 and MSH6. Cancer Res. 1997 Sep 15;57(18):3949-55.

Valentinis B, Zaffaroni N, Sturani E, Martegani E, Silvestrini R. Detection of DNA polymerase-beta gene expression by competitive polymerase chain reaction in human ovarian carcinoma cells. Anticancer Res. 1993 Jan-Feb;13(1):125-8.

van Duin M, de Wit J, Odijk H, Westerveld A, Yasui A, Koken HM, Hoeijmakers JH, Bootsma D. Molecular characterization of the human excision repair gene ERCC-1: cDNA cloning and amino acid homology with the yeast DNA repair gene RAD10. Cell. 1986 Mar 28;44(6):913-23.

van Vuuren AJ. Nucleotide Excision Repair : ERCC1 and TFHII Complexes. 1995. Thesis Erasmus University Rotterdam, The Netherlands.

Vendetti JM. The National Cancer Institute antitumor drug discovery program: current and future perspectives: a commentary. Cancer Treat Rep. 1983; 67:767-74.

Vikhanskaya F, Colella G, Valenti M, Parodi S, D'Incalci M, Broggini M. Cooperation between p53 and hMLH1 in a human colocarcinoma cell line in response to DNA damage. Clin Cancer Res. 1999 Apr;5(4):937-41.

Vilpo JA, Vilpo LM, Szymkowski DE, O'Donovan A, Wood RD. An XPG DNA repair defect causing mutagen hypersensitivity in mouse leukemia L1210 cells. Mol Cell Biol. 1995 Jan;15(1):290-7.

Visse R. DNA Repair by the Escherichia Colu UvrABC Endonuclease. 1994. Thesis University of Leiden, The Netherlands.

Vogelstein B, Kinzler KW. p53 function and dysfunction. Cell. 1992 Aug 21;70(4):523-6.

Waga S, Hannon GJ, Beach D, Stillman B. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature. 1994 Jun 16;369(6481):574-8.

Wagner AJ, Kokontis JM, Hay N. Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21wafl/cipl. Genes Dev. 1994 Dec 1;8(23):2817-30.

Walton MI, Whysong D, O'Connor PM, Hockenbery D, Korsmeyer SJ, Kohn KW. Constitutive expression of human Bcl-2 modulates nitrogen mustard and camptothecin induced apoptosis. Cancer Res. 1993 Apr 15;53(8):1853-61.

Wang AL, Tew KD. Increased glutathione-S-transferase activity in a cell line with acquired resistance to nitrogen mustards. Cancer Treat Rep. 1985 Jun;69(6):677-82.

Wang Y, Cortez D, Yazdi P, Neff N, Elledge SJ, Qin J. BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. Genes Dev. 2000 Apr 15;14(8):927-39.

Wang YY, Teicher BA, Shea TC, Holden SA, Rosbe KW, al-Achi A, Henner WD. Cross-resistance and glutathione-S-transferase-pi levels among four human melanoma cell lines selected for alkylating agent resistance. Cancer Res. 1989 Nov 15;49(22):6185-92.

Weiland KL, Dooley TP. In vitro and in vivo DNA bonding by the CC-1065 analogue U-73975. Biochemistry. 1991 Jul 30;30(30):7559-65.

Weinert TA, Hartwell LH. The RAD9 gene controls the cell cycle response to DNA damage in Saccharomyces cerevisiae. Science. 1988 Jul 15;241(4863):317-22.

West SC. Enzymes and molecular mechanisms of genetic recombination. Annu Rev Biochem. 1992;61:603-40.

Wildenberg J, Meselson M. Mismatch repair in heteroduplex DNA. Proc Natl Acad Sci U S A. 1975 Jun;72(6):2202-6.

Wilson AP. Characterization of a cell line derived from the ascites of a patient with papillary serous cystadenocarcinoma of the ovary. J Natl Cancer Inst. 1984 Mar;72(3):513-21.

Wood RD. DNA repair in eukaryotes. Annu Rev Biochem. 1996;65:135-67.

Wooster R, Bignell G, Lancaster J, Swift S, Seal S, Mangion J, Collins N, Gregory S, Gumbs C, Micklem G. Identification of the breast cancer susceptibility gene BRCA2. Nature. 1995 Dec 21-28;378(6559):789-92.

Wooster R, Cleton-Jansen AM, Collins N, Mangion J, Cornelis RS, Cooper CS, Gusterson BA, Ponder BA, von Deimling A, Wiestler OD, et al. Instability of short tandem repeats (microsatellites) in human cancers. Nat Genet. 1994 Feb;6(2):152-6.

Woynarowski JM, Chapman WG, Napier C, Herzig MC, Juniewicz P. Sequence- and region-specificity of oxaliplatin adducts in naked and cellular DNA. Mol Pharmacol. 1998 Nov;54(5):770-7.

Woynarowski JM, Faivre S, Herzig MC, Arnett B, Chapman WG, Trevino AV, Raymond E, Chaney SG, Vaisman A, Varchenko M, Juniewicz PE. Oxaliplatin-induced damage of cellular DNA Mol Pharmacol. 2000 Nov;58(5):920-7.

Wu PK, Qu Y, Van Houten B, Farrell N. Chemical reactivity and DNA sequence specificity of formally monofunctional and bifunctional bis(platinum) complexes. J Inorg Biochem. 1994 May 15;54(3):207-20.

Wu X, Bayle JH, Olson D, Levine AJ. The p53-mdm-2 autoregulatory feedback loop. Genes Dev. 1993 Jul;7(7A):1126-32.

Wu X, Levine AJ. p53 and E2F-1 cooperate to mediate apoptosis. Proc Natl Acad Sci U S A. 1994 Apr 26;91(9):3602-6.

Wyllie AH. Apoptosis (the 1992 Frank Rose Memorial Lecture). Br J Cancer. 1993 Feb;67(2):205-8.

Yamada M, O'Regan E, Brown R, Karran P. Selective recognition of a cisplatin-DNA adduct by human mismatch repair proteins. Nucleic Acids Res. 1997 Feb 1;25(3):491-6.

Yamamoto H, Sawai H, Weber TK, Rodriguez-Bigas MA, Perucho M. Somatic frameshift mutations in DNA mismatch repair and proapoptosis genes in hereditary nonpolyposis colorectal cancer. Cancer Res. 1998 Mar 1;58(5):997-1003.

Yarbo JW. Carboplatin (JM8) Update: Current Perspectives and Future Directions. Seminars in Oncology.1992; 19 (Supp 2):1-164.

Yin Y, Tainsky MA, Bischoff FZ, Strong LC, Wahl GM. Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. Cell. 1992 Sep 18;70(6):937-48.

Yonish-Rouach E, Resnitzky D, Lotem J, Sachs L, Kimchi A, Oren M. Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature. 1991 Jul 25;352(6333):345-7.

Yu JJ, Mu C, Dabholkar M, Guo Y, Bostick-Bruton F, Reed E.Alternative splicing of ERCC1 and cisplatin-DNA adduct repair in human tumor cell lines. Int J Mol Med. 1998 Mar;1(3):617-20.

Zambetti GP, Levine AJ. A comparison of the biological activities of wild-type and mutant p53. FASEB J. 1993 Jul;7(10):855-65.

Zamble DB, Lippard SJ. Cisplatin and DNA repair in cancer chemotherapy. Trends Biochem Sci. 1995 Oct;20(10):435-9.

Zamzami N, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T, Susin SA, Petit PX, Mignotte B, Kroemer G. Sequential reduction of mitochondrial

transmembrane potential and generation of reactive oxygen species in early programmed cell death. J Exp Med. 1995 Aug 1;182(2):367-77.

Zeller WJ, Fruhauf S, Chen G, Keppler BK, Frei E, Kaufmann M. Chemoresistance in rat ovarian tumours. Eur J Cancer. 1991;27(1):62-7.

Zhan Q, Alamo I, Yu K, Boise LH, Cherney B, Tosato G, O'Connor PM, Fornace AJ. The apoptosis-associated gamma-ray response of BCL-X(L) depends on normal p53 function. Oncogene. 1996 Nov 21;13(10):2287-93.

Zhan Q, Carrier F, Fornace AJ. Induction of cellular p53 activity by DNA-damaging agents and growth arrest. Mol Cell Biol. 1993 Jul;13(7):4242-50.

Zhan Q, Fan S, Bae I, Guillouf C, Liebermann DA, O'Connor PM, Fornace AJ. Induction of bax by genotoxic stress in human cells correlates with normal p53 status and apoptosis. Oncogene. 1994 Dec;9(12):3743-51.

Zhang H, Somasundaram K, Peng Y, Tian H, Zhang H, Bi D, Weber BL, El-Deiry WS. BRCA1 physically associates with p53 and stimulates its transcriptional activity. Oncogene. 1998 Apr 2;16(13):1713-21.

Zhang JG, Lindup WE. Cisplatin nephrotoxicity: decreases in mitochondrial protein sulphydryl concentration and calcium uptake by mitochondria from rat renal cortical slices. Biochem Pharmacol. 1994 Mar 29;47(7):1127-35.

Zhen W, Link CJ, O'Connor PM, Reed E, Parker R, Howell SB, Bohr VA. Increased gene-specific repair of cisplatin interstrand cross-links in cisplatin-resistant human ovarian cancer cell lines. Mol Cell Biol. 1992 Sep;12(9):3689-98.

Zou Y, Van Houten B, Farrell N. Sequence specificity of DNA-DNA interstrand cross-link formation by cisplatin and dinuclear platinum complexes. Biochemistry. 1994 May 10;33(18):5404-10.

Zunino F, Perego P, Pilotti S, Pratesi G, Supino R, Arcamone F. Role of apoptotic response in cellular resistance to cytotoxic agents. Pharmacol Ther. 1997 Oct-Dec;76(1-3):177-85.